Supplementary Materials for

Albumin Nanoparticle Encapsulation of Potent Cytotoxic Therapeutics Shows Sustained Drug Release and Alleviates Cancer Drug Toxicity

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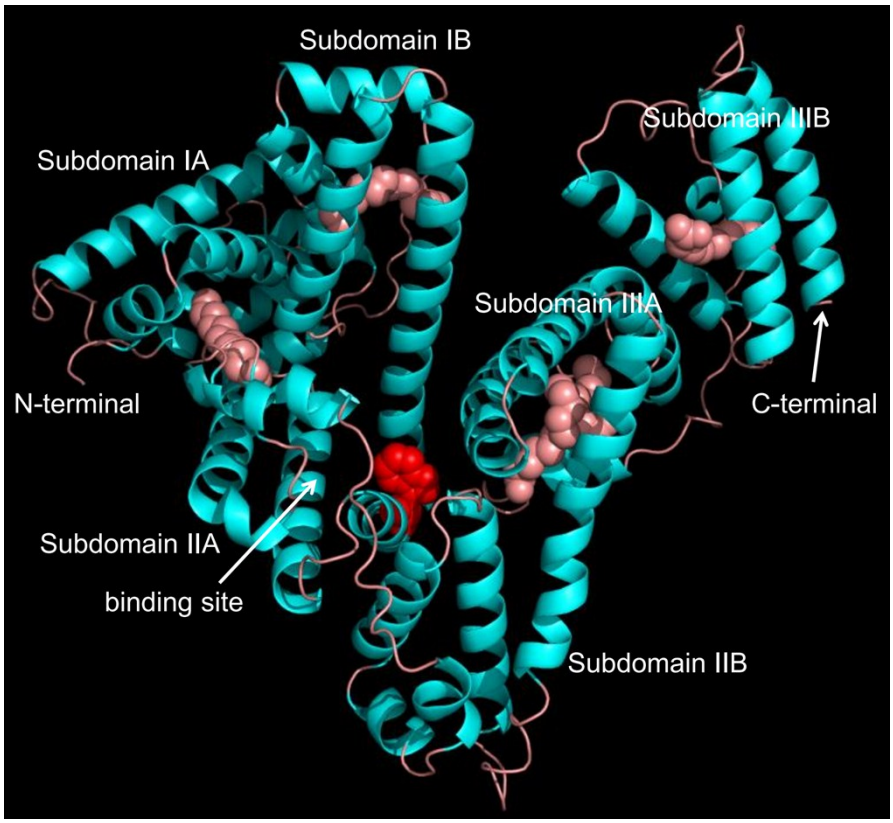


Figure S1 Crystal structure of human serum albumin (HSA) (pdb-entry 1BJ5) complexed with five molecules of myristic acid (brown). The tryptophan residue (Trp-214) is indicated as red.

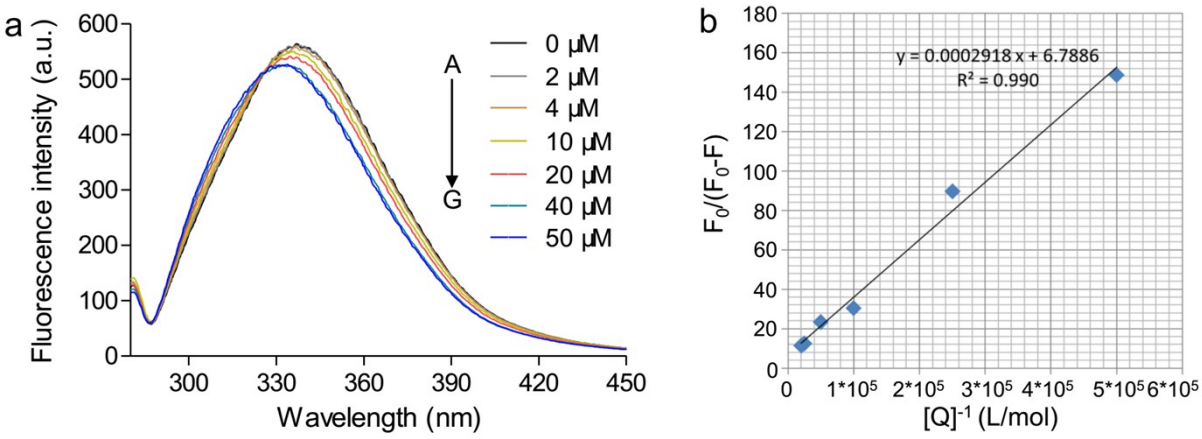


Figure S2 (**a**) Fluorescence spectral change of HSA solution upon the addition of paclitaxel (PTX). Measurement conditions: 5 μM HSA in PBS, pH 7.4, 25°C, and λ_{ex}=280 nm; PTX was added 0-50 μM (from A to G). (**b**) The modified Stern-Volmer plot for the fluorescence quenching of HSA by PTX at 25°C (λ_{em}=337 nm); [Q] represents the concentration of PTX.

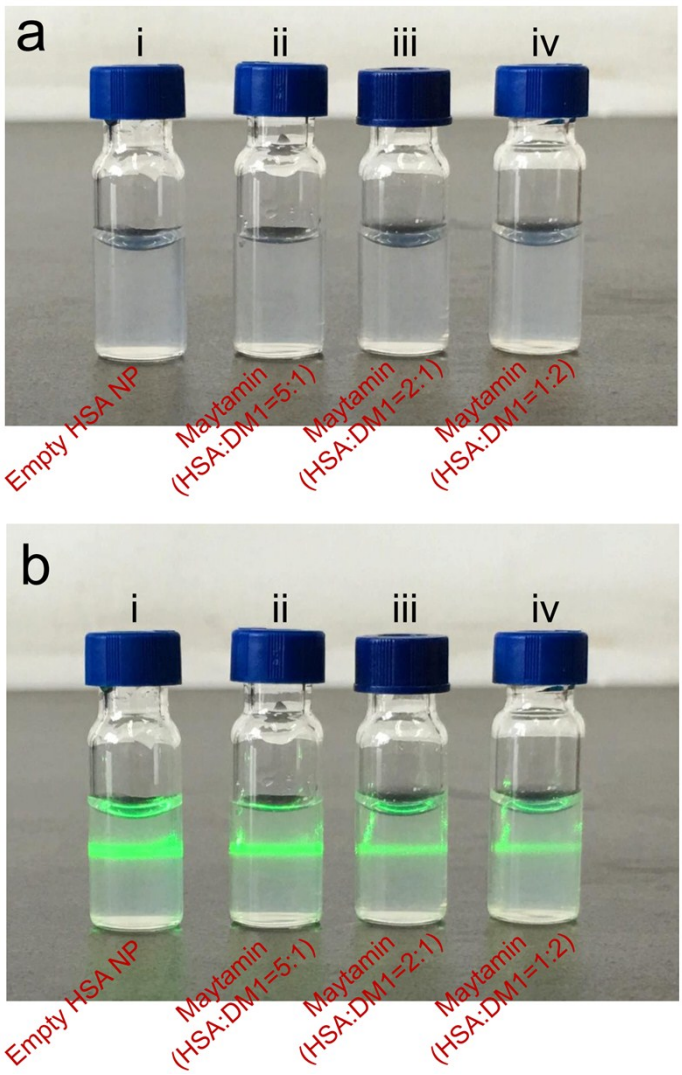


Figure S3 (**a**) Photographs of empty HSA NP (0.2 mg/mL) and maytamins (at 0.2 mg/mL of HSA concentrations) that were fabricated at different molar ratios of HSA to DM1. The samples were prepared according to the protocol described in the experimental section and re-suspended in deionized (DI) water. The feed ratios of HSA to DM1 in ii, iii, and iv were 5:1, 2:1, and 1:2, respectively. (**b**) When a green laser beam was passed through the samples as indicated in (**a**), laser scattering was observed due to the presence of HSA-assembled nanoparticles. This result is in agreement with the TEM images and DLS analysis, suggesting the formation of maytamins.

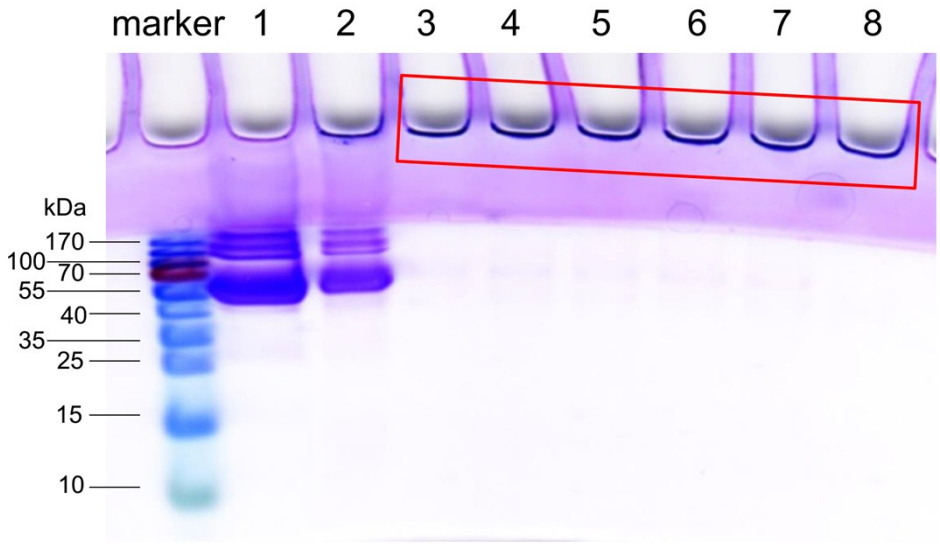


Figure S4 SDS-PAGE analysis of HSA-assembled nanoparticles. Lane 1, native HSA; Lane 2, HSA NPs prepared without cross-linker glutaraldehyde (GA); Lane 3, empty HSA NPs; Lanes 4 to 8 represent maytamins prepared at varying molar ratios of HSA to DM1, namely 5:1, 2:1, 1:1, 1:2, and 1:5 for lane 4, 5, 6, 7, and 8, respectively. The monomeric HSA (Mw = 67 kDa) disappeared due to the cross-linking by GA, and the cross-linked HSA NPs remained at the top of the gel (red box). In contrast, when HSA NPs were prepared in the absence of GA (lane 2), non-covalently assembled HSA NPs dissociated into monomers in the electrophoresis gel.

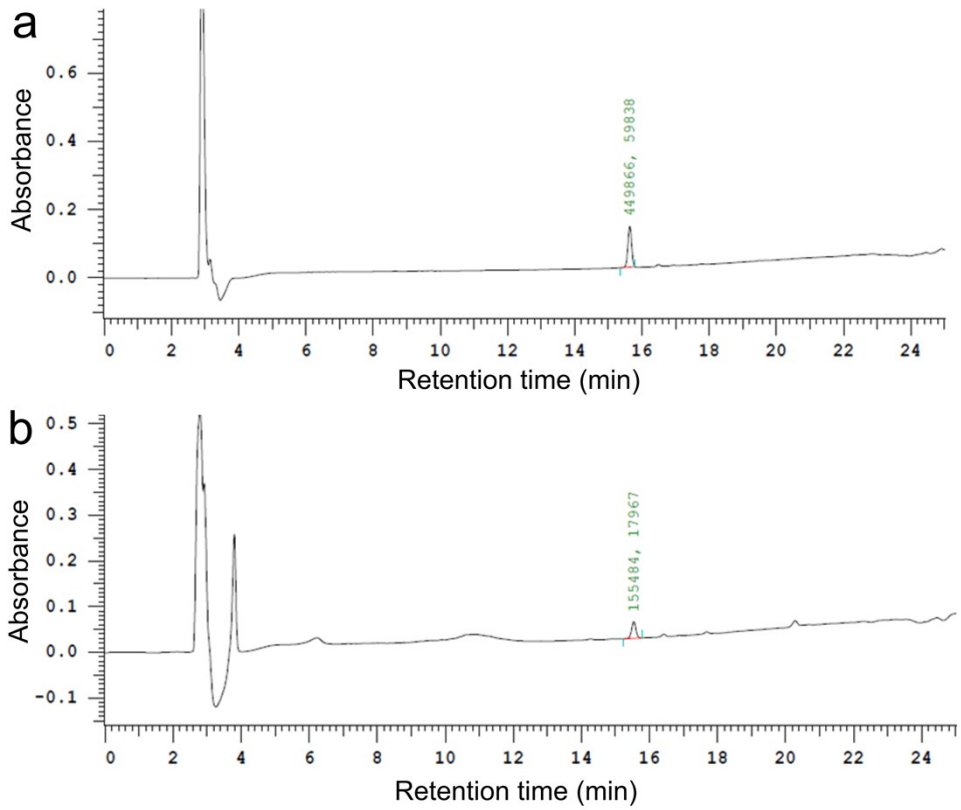


Figure S5 The total amount of DM1 (a) and the unencapsulated DM1 in supernatants determined by reverse-phase HPLC analysis. The DM1 molecules in supernatant were extracted with excess acetonitrile (H_2O/CH_3CN, v/v, 1/2) and subjected to analytical HPLC. For HPLC analysis, YMC-Pack ODS-A analytical column (5 μm, 250 × 4.6 mm) was used. The mobile phase was a gradient of acetonitrile (solvent A) and water (solvent B) at a flow rate of 1.0 mL/min. The linear gradient was 20% A: 80% B to 100%A: 0% B within 20 min. UV detection was monitored at 220 nm.

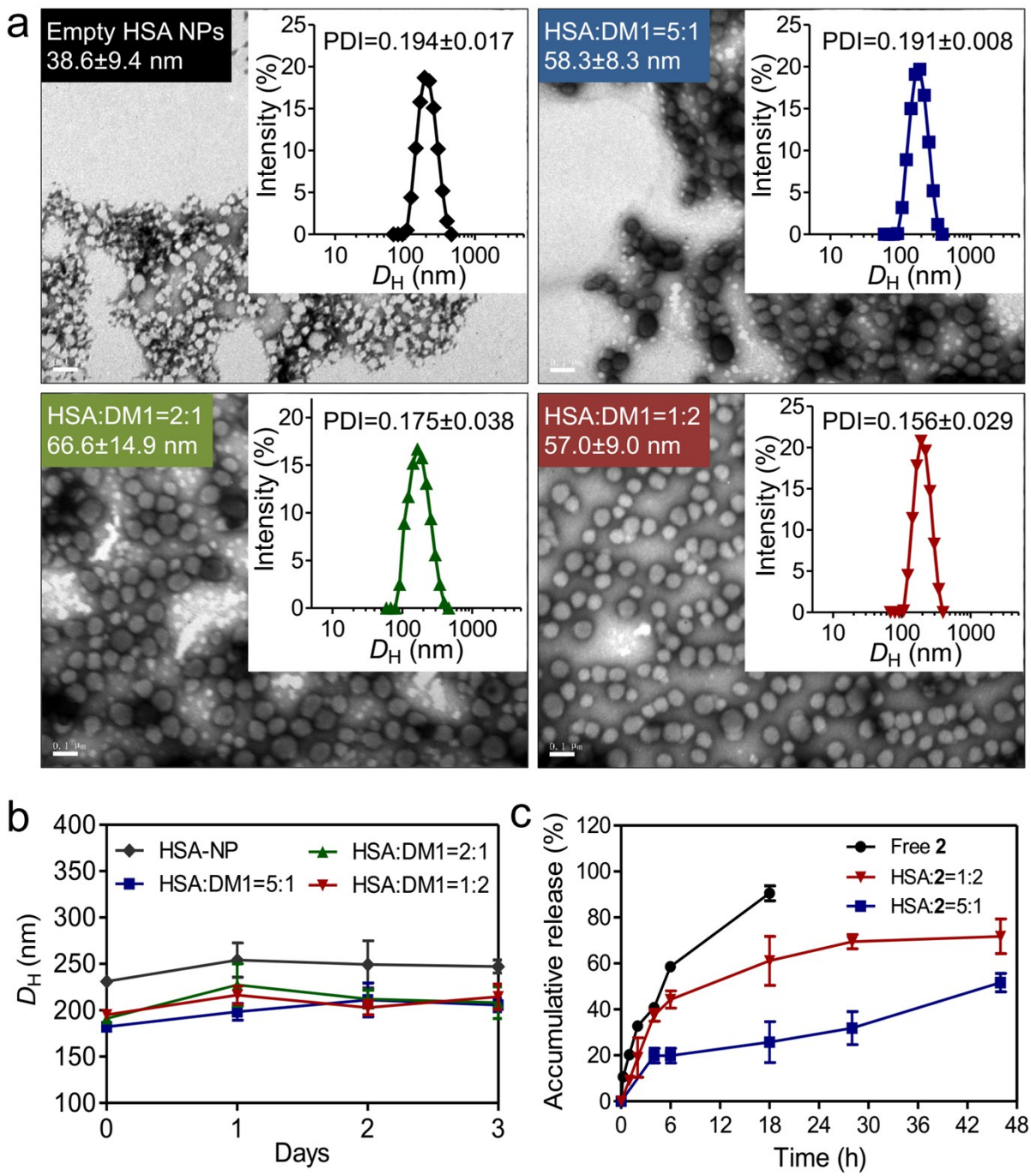


Figure S6 (**a**) TEM images of empty HSA nanoparticles and maytamins prepared by the different feed ratios of HSA and DM1; scale bars: 100 nm; Inset: D_H distribution characterized by DLS. (**b**) Stability of empty NPs and maytamins in phosphate buffered saline (PBS, pH 7.4) at 4°C. (**c**) *In vitro* release profiles of Cy3-labeled DM1 from maytamins fabricated with indicated molar ratios of HSA to DM1 using a dialysis method over a 46-h period against PBS (pH 7.4) at 37°C. The data are presented as the means ± SD (n=3).

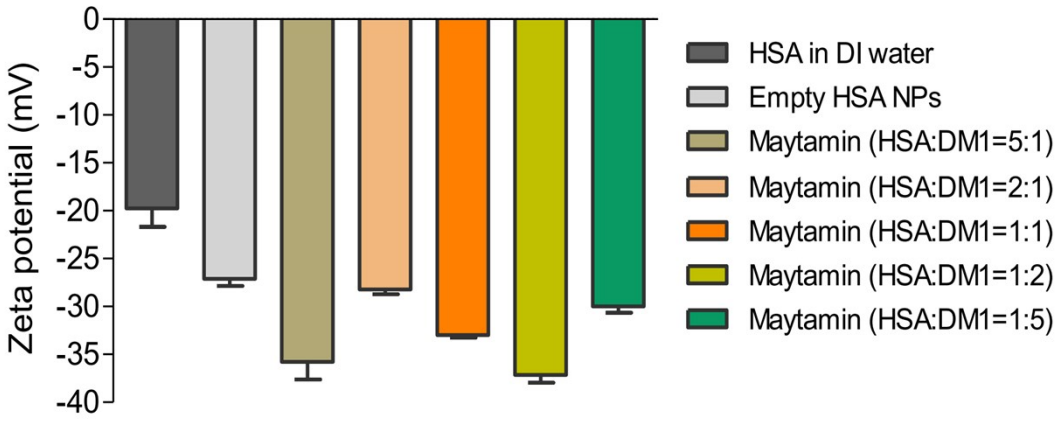


Figure S7 Zeta potentials of HSA solution and maytamins fabricated at different molar ratios (at 0.2 mg/mL of HSA concentrations).

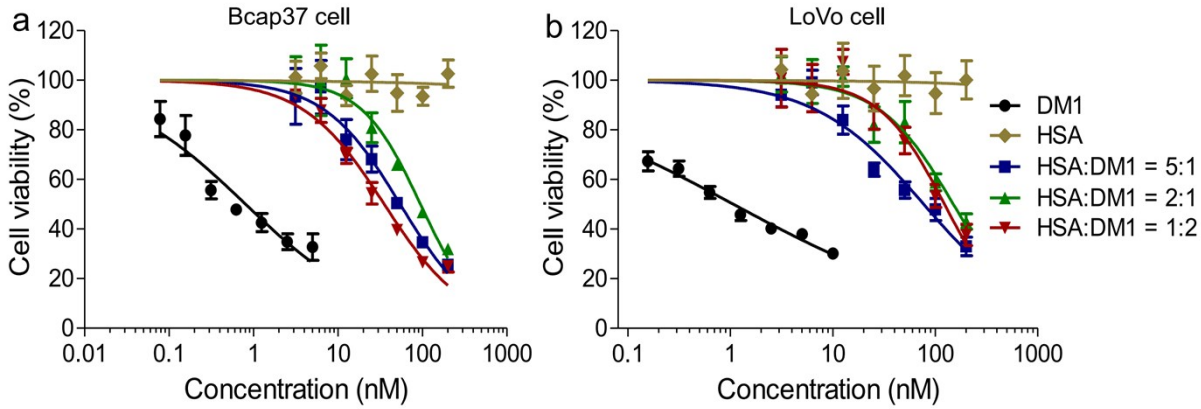


Figure S8 The cell viability in Bcap37 (**a**) and LoVo (**b**) cells after 72 h treatment with free DM1, empty HSA NPs, and maytamins, as measured by standard MTT assay (the data are presented as the means ± SD, n=4).

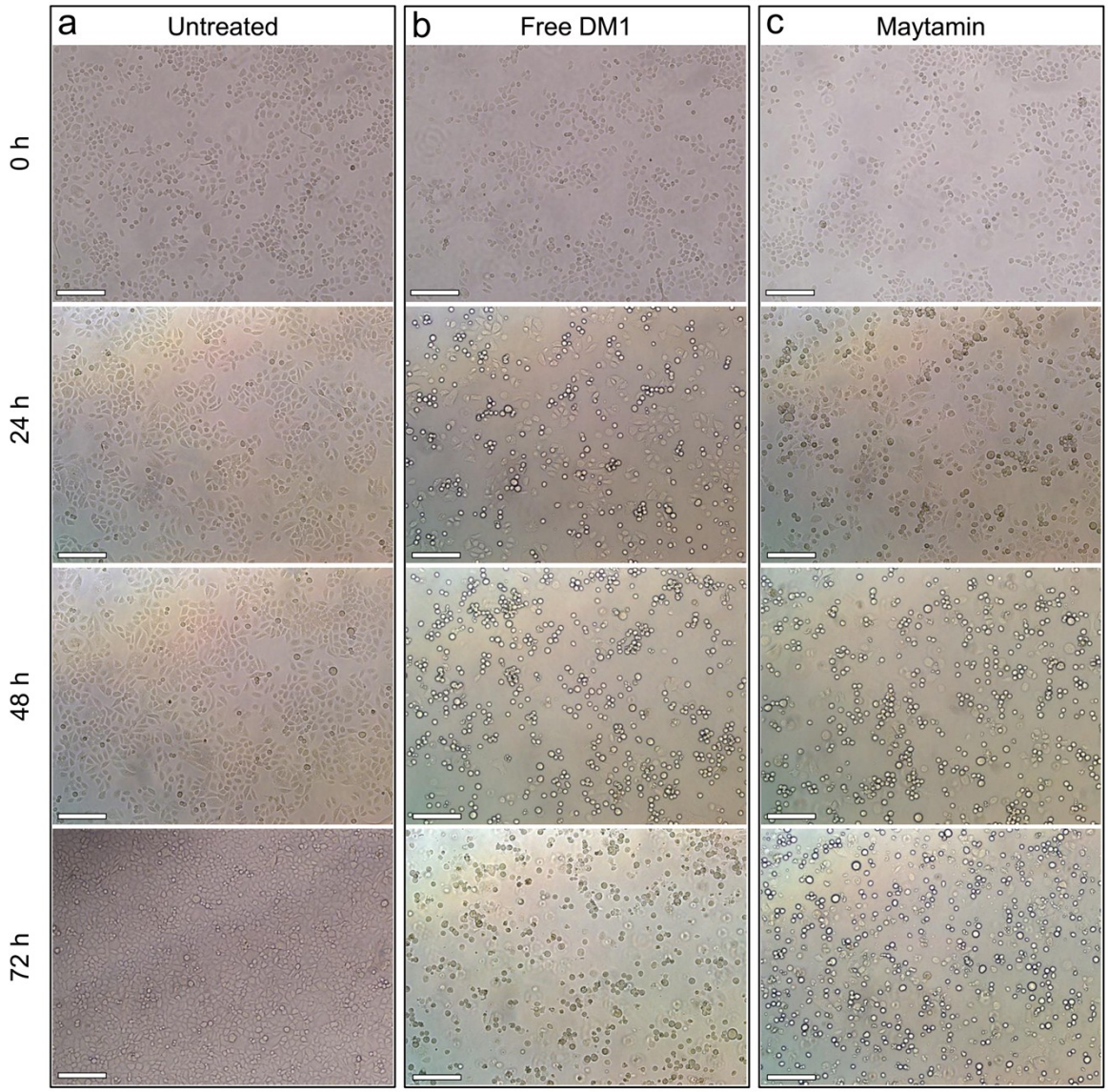


Figure S9 Morphology changes of breast cancer Bcap37 cells when the cells were incubated with free DM1 (25 nM) and maytamin at 25 nM of DM1 equivalent concentration (HSA:DM1=5:1). Untreated cells were used as control. Scale bars: 200 μm. Upon treatment with maytamin, Bcap37 cells exhibited apoptosis-associated morphology changes such as cell shrinkage and detachment from the culture plate. This result indicate that DM1 can be released from HSA-assembled NP and exert cell killing effect.

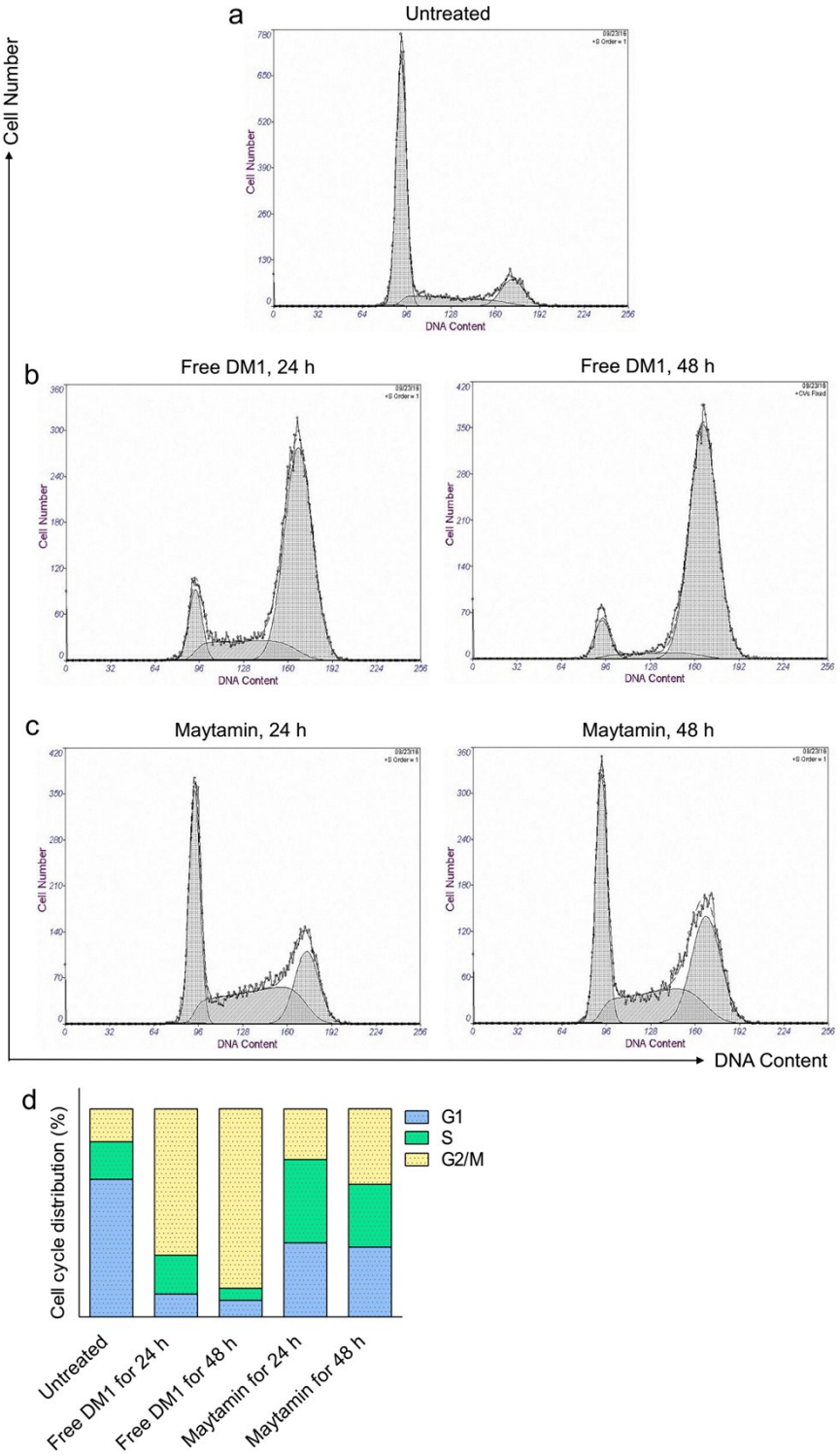


Figure S10 Flow cytometric analysis of cell cycle of Bcap37 cells. The cells were treated with free DM1 (25 nM, **b**) and maytamin at the concentration of 25 nM (DM1 equivalent, **c**). Untreated cells were used as a control (**a**). (**d**) Changes of cell cycle distribution. Abbreviations: G1, Gap 1 phase; S, Synthesis phase; G2/M, Gap 2/Mitosis phase. DM1 is microtubule-targeted drug by depolymerizing microtubules and can arrest cells in mitosis.[S1] Similar to the treatement with free DM1, DM1-encapsulated maytamins arrested the cell cycle of Bcap37 cells at the G2/M phase.

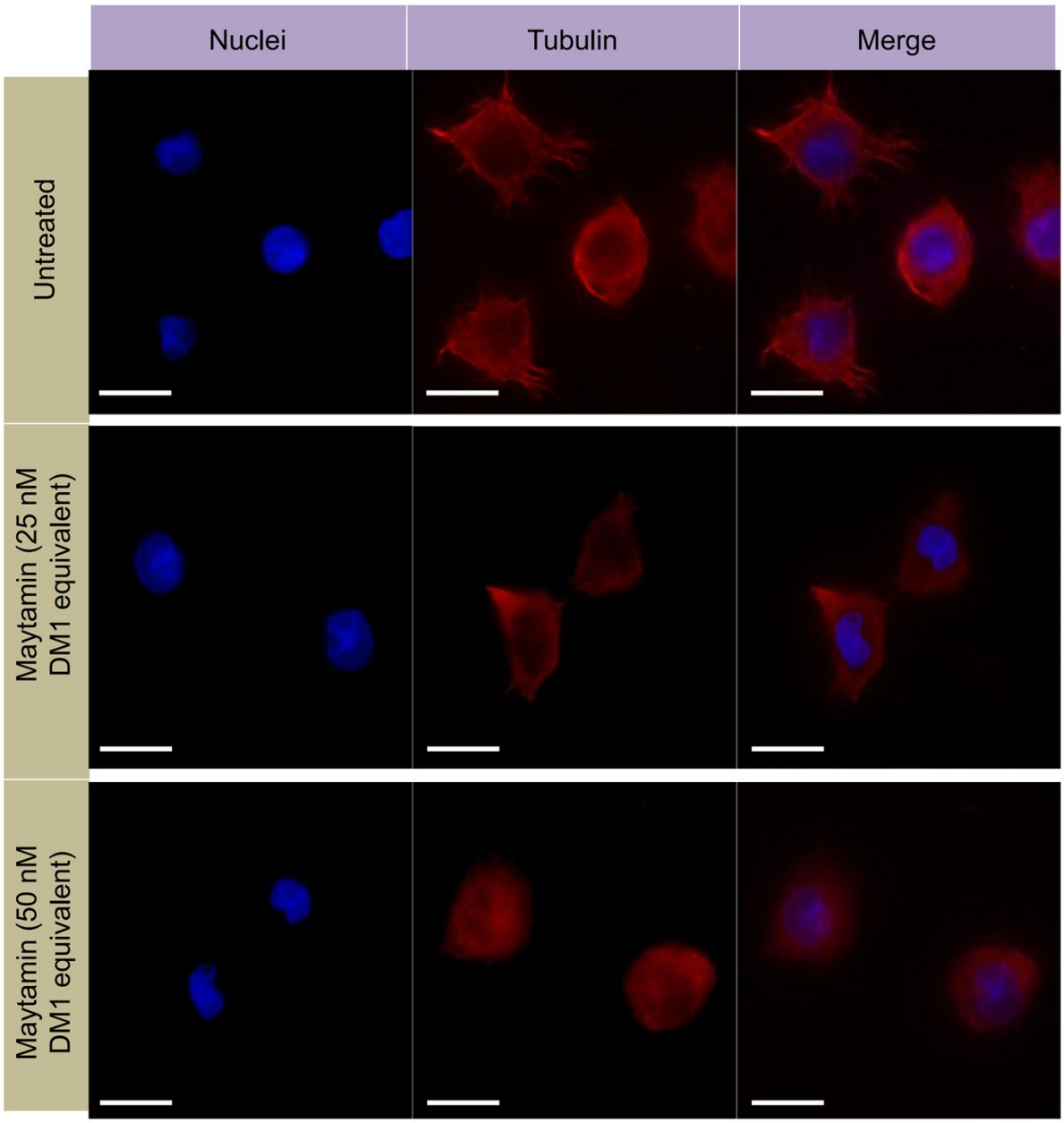


Figure S11 α-Tubulin immunodetection by CLSM in Bcap37 cells. The cells were treated with DMSO or maytamins containing 25 or 50 nM DM1 agents. After incubation with these nanoformulations for 12 h, the cells were fixed and stained with anti-α-tubulin antibody. Scale bars: 30 μm.

Table S1 Antitumor activity of maytamin against human cancer Bcap37 and LoVo cells expressed as IC_{50} (nM) [a]

	Free DM1	HSA:DM1=5:1[b]	HSA:DM1=2:1[b]	HSA:DM1=1:2[b]	Empty HSA NP
Bcap37 cell	0.81±0.13	54.5±7.8	94.7±12.6	35.3±3.0	N.E.
LoVo cell	1.07±0.13	77.1±9.2	149.9±21.9	122.8±15.9	N.E.

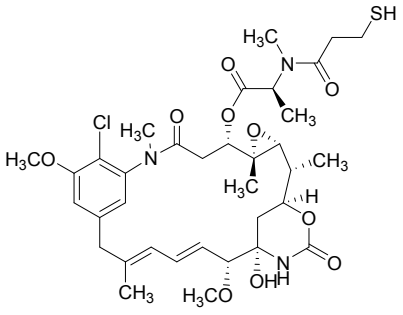
[a] Determined by MTT assay; N.E., not effective.

[b] Molar ratio was used to prepare DM1-loaded HSA NPs.

General materials and methods

The thiolated maytansine derivative DM1 (Mw = 738.28) was purchased from BrightGene (Suzhou, China) and confirmed by our lab using ^{1}H NMR and mass spectra. Human serum albumin (Mw = 67 kDa) was purchased from Sigma-Aldrich (USA). Cyanine3 maleimide was purchased from Lumiprobe (Florida, USA). Glutaraldelyde and all other compounds and solvents were purchased from J&K Chemical Ltd. (Shanghai, China).

All reactions were performed in a dry atmosphere. ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker 400 (400 MHz) spectrometer and calibrated to the residual solvent peak or tetramethylsilane (= 0 ppm). Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, dt = double triplet, br = broad. High-resolution mass spectrometry (HRMS)-ESI was recorded on AB TripleTOF 5600^{plus} System (AB SCIEX, Framingham, USA) instruments. Reverse phase high-performance liquid chromatography (RP-HPLC) was carried out on a Hitachi Chromaster 5000 system. Compounds were purified using a YMC-Pack ODS-A column (5 μm, 250 × 10 mm) or their purities were analyzed by a YMC-Pack ODS-A column (5 μm, 250 × 4.6 mm). All HPLC runs used linear gradients of acetonitrile (solvent A) and water (solvent B) containing 0.1% trifluoroacetic acid (TFA).



DM1

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.82 (d, 1H, *J* = 1.6 Hz), 6.72 (d, 1H, *J* = 11.1 Hz), 6.67 (d, 1H, *J* = 1.6 Hz), 6.44 (dd, 1H, *J* = 15.3, 11.1 Hz), 6.21 (s, 1H), 5.64 (dd, 1H, *J* = 15.3, 9.0 Hz), 5.42 (q, 1H, *J* = 6.8 Hz), 4.77 (dd, 1H, *J* = 12.0, 3.0 Hz), 4.27 (dd, 1H, *J* = 16.5, 6.1 Hz), 3.98 (s, 3H), 3.59 (dd ,2H, *J* = 67.8, 10.9 Hz), 3.36 (s, 3H), 3.21 (s, 3H), 3.07 (dd, 2H, *J* = 32.9, 11.1 Hz), 2.85 (s, 3H), 2.82 – 2.51 (m, 5H), 2.19 (dd, 1H, *J* = 14.3, 3.0 Hz), 1.65 (s, 3H), 1.59 (s, 2H), 1.47 (m, 1H), 1.31 (t, 6H, *J* = 6.8 Hz), 0.80 (s, 3H).

HRMS: calcd for $[C_{35}H_{49}ClN_3O_{10}S]^+$ $[M+H]^+$ = 738.2822; obsd = 738.2817.

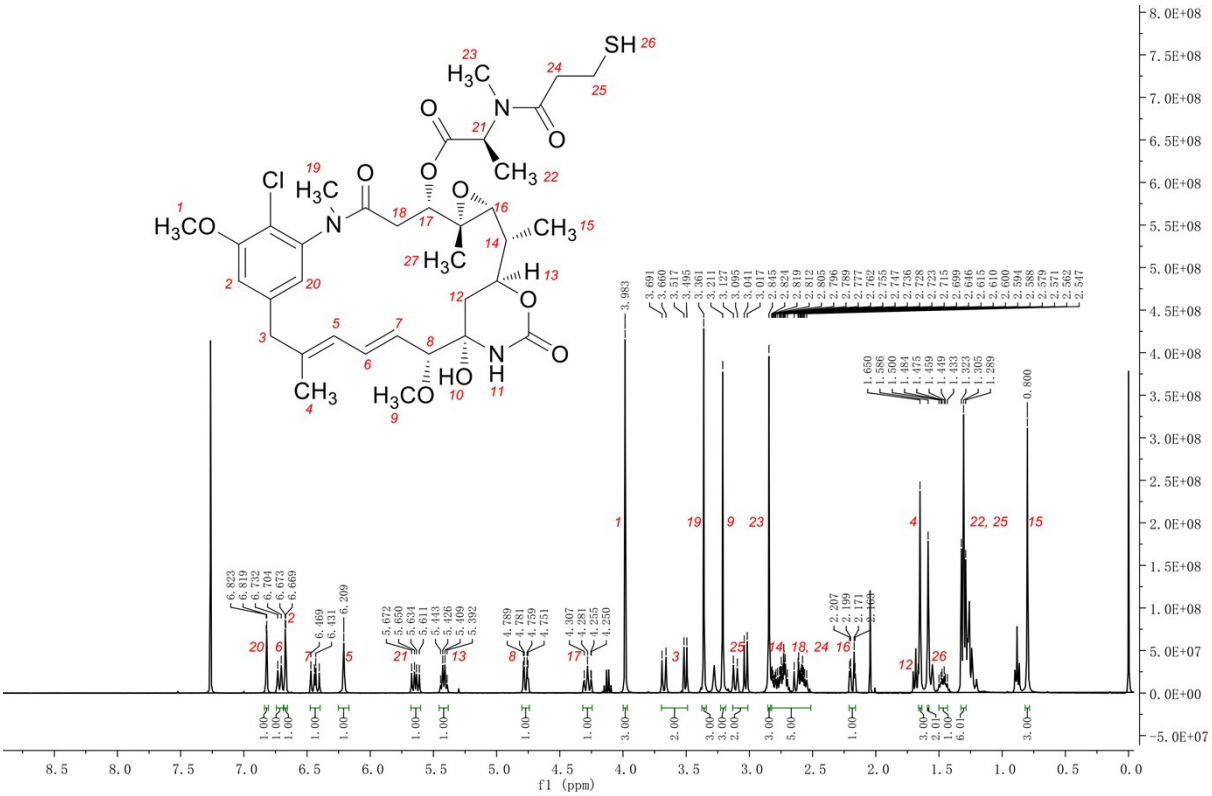


Figure S12 ^{1}H NMR spectrum of thiolated maytansine derivative DM1 in $CDCl_3$.

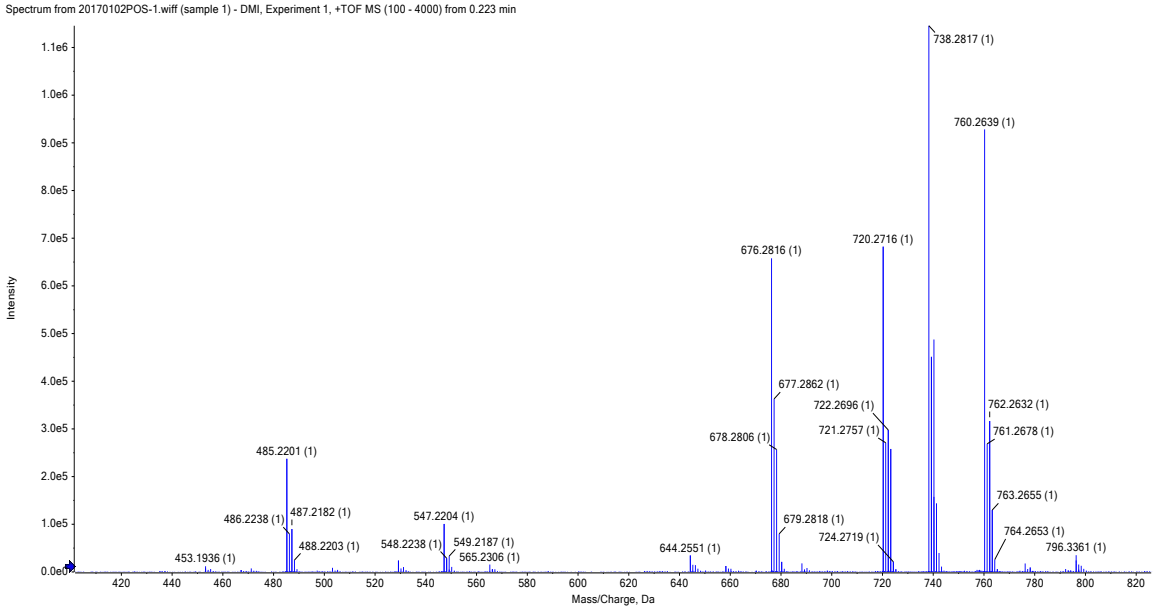
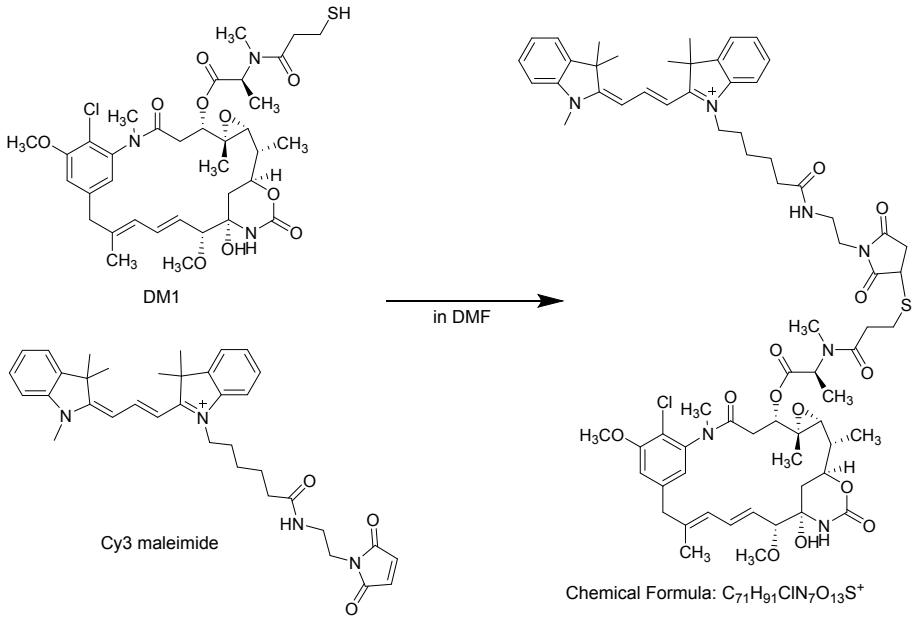


Figure S13 High-resolution mass spectrum of DM1.

Synthesis of compound 2



Synthetic scheme S1

To a solution of maytansine derivative DM1 (10.6 mg, 14 μmol) in 1 mL of anhydrous DMF was added Cyanine3 maleimide (8 mg, 13 μmol). The reaction mixture was stirred at 37°C for 4 h. After removing the solvent, the residue was purified by RP-HPLC to give compound **2** (8 mg, 47%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.72 (s, 1H), 7.42 (td, 2H, *J* = 7.5, 3.5 Hz), 7.38 (d, 1H, *J* = 2.2 Hz), 7.36 (d, 1H, *J* = 2.3 Hz), 7.28 (s, 2H), 7.16 (d, 2H, *J* = 7.8 Hz), 6.83 (d, 1H, *J* = 6.8 Hz), 6.70 – 6.63 (m, 2H), 6.39 (m, 4H), 5.66 (dd, 1H, *J* = 15.2, 6.7 Hz), 5.39 – 5.31 (m, 1H), 4.81 – 4.76 (m, 1H), 4.28 (t, 1H, *J* = 11.1 Hz), 4.04 (s, 2H), 3.98 (d, 3H, *J* = 2.7 Hz), 3.64 (t, 7H, *J* = 17.7 Hz), 3.49 (d, 2H, *J* = 8.4 Hz), 3.35 (d, 3H, *J* = 6.2 Hz), 3.20 (s, 3H), 3.13 – 3.06 (m, 5H), 2.85 (s, 3H), 2.66 – 2.57 (m, 3H), 2.21 (d, 3H, *J* = 19.2 Hz), 1.73 (d, 9H, *J* = 4.3 Hz), 1.61 (dd, 6H, *J* = 21.6, 10.8 Hz), 1.49 – 1.44 (m, 2H), 1.36 – 1.28 (m, 9H), 1.27 (s, 3H), 1.25 – 1.23 (m, 2H), 0.79 (s, 3H).

HRMS: calcd for $[C_{71}H_{91}ClN_7O_{13}S]^+$ = 1316.6079; obsd = 1316.6026.

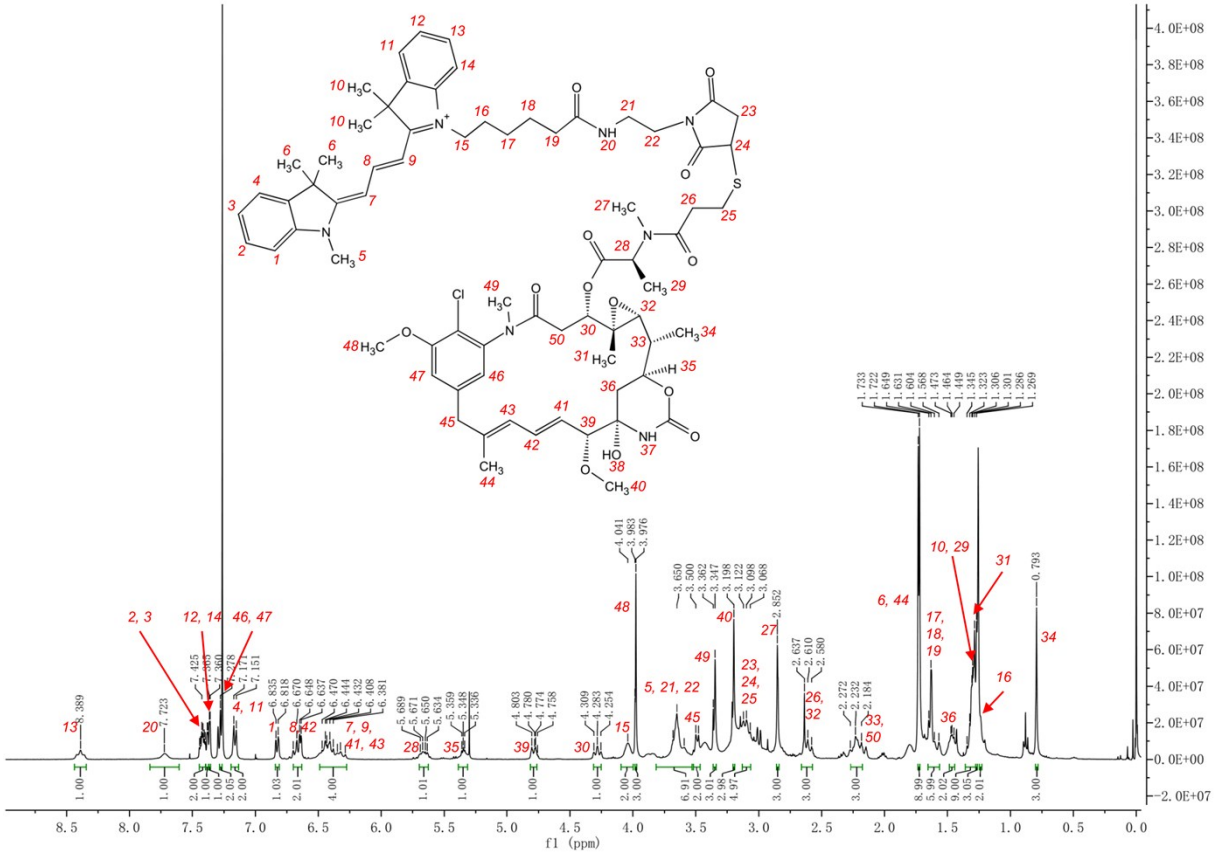


Figure S14 ^{1}H NMR spectrum of Cy3-labeled DM1 compound **2** in $CDCl_3$.

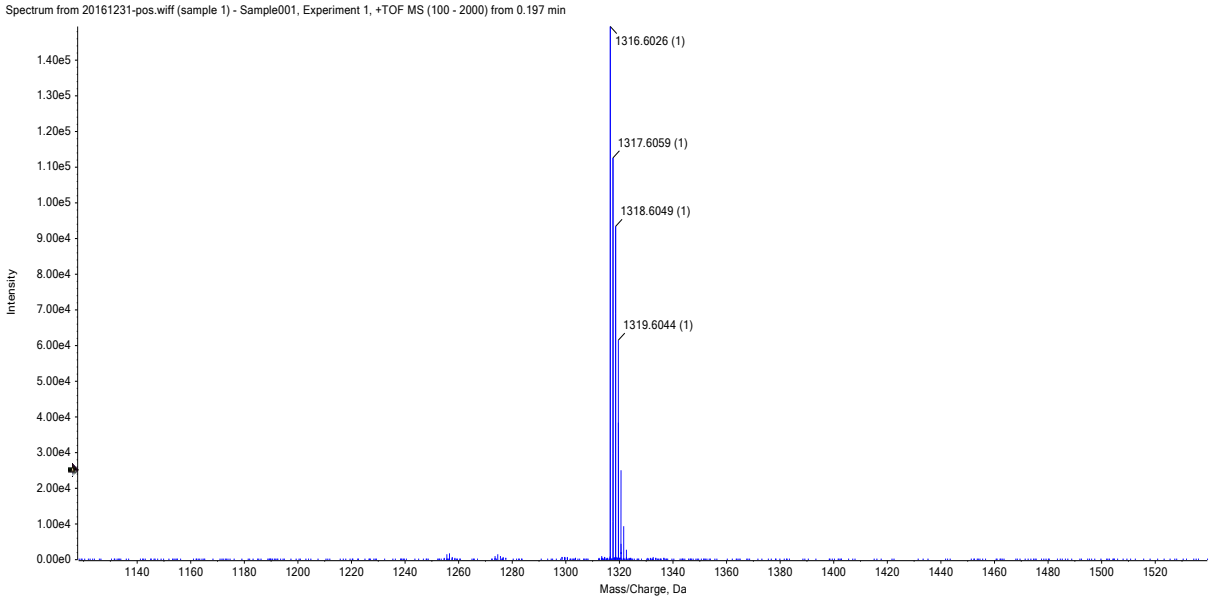


Figure S15 High-resolution mass spectrum of compound **2**.

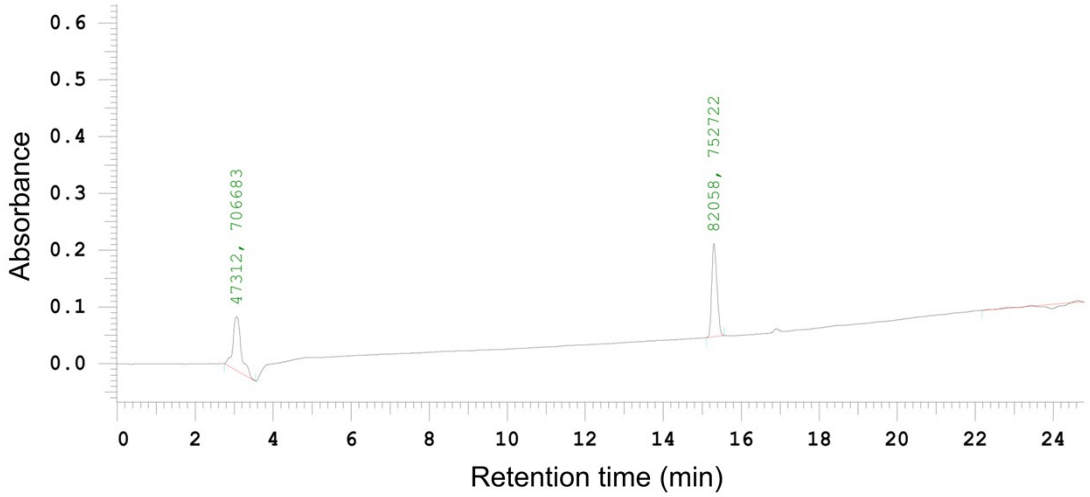


Figure S16 Reverse-phase HPLC chromatogram of compound **2**. The purified **2** was subjected to analytical HPLC using a C18 reverse-phase column (5 μm, 250 mm × 4.6 mm). A gradient of 20-100% acetonitrile in water within 20 min was adopted as the mobile phase at a flow rate of 1 mL/min together with UV detection at a wavelength of 220 nm.

Preparation of drug-loaded HSA nanoparticles

Various maytamins formulating DM1 were prepared by a desolvation method. Briefly, predetermined amounts of DM1 (Mw = 738.29, 5 mg/mL in DMSO) and 1 mL of HSA solution (Mw = 67 kDa, 10 mg/mL in DI water) were stirred for 30 min. After then, 3 mL of ethanol was added dropwise (1 mL/min) to the above solution with stirring (500 rpm). The mixture was added cross-linker glutaraldehyde (GA, 8%, 10 μL) and then stirred for 12 h at 25°C. Finally, the NPs were precipitated using ultracentrifuge at 50,000 *g* for 45 min to remove free HSA, unencapsulated DM1 and glutaraldehyde, and washed three times with DI water to obtain maytamins.

Fluorescence titration to evaluate the association constant

Fluorescence emission spectra were recorded on a Shimadzu spectrofluorometer (Shimadzu RFPC-5301) using a 1.0 cm path length quartz cuvette. 2 mL of HSA solution (5 μM in PBS, pH 7.4) was added by compounds (i.e., DM1 and PTX in DMSO, 5 mM) with a microsyringe. The solutions then were gently mixed and allowed to stand for 10 min to equilibrate. The fluorescence spectra were measured upon excitation at 280 nm.

The modified Stern-Volmer plot (λ_{em}=337 nm) was used to evaluate the association constant for drug-HSA interaction:

$F_0/(F_0\text{-}F)=1/f_aK_a[Q]+1/f_a$

where K_a is the modified Stern-Volmer association constant, which is the quotient of an ordinate $1/f_a$ and slope $1/f_aK_a$ for the accessible fluorophores, and f_a is the fraction of accessible fluorescence. [Q] represents the final concentration of added drugs. F_0 and F represent the initial fluorescence intensity of HSA solution and the fluorescence intensities upon addition of drugs, respectively.

In the case of DM1, plotting $F_0/(F_0\text{-}F)$ with 1/[Q] yielded the equation, y=0.0001059x+2.2586, as indicated in Figure 1d. We thus obtained that $1/f_a$ and $1/f_aK_a$ were 2.2586 and 0.0001059, respectively. Furthermore, we concluded that f_a = 0.44275, and K_a = 2.1×10^4 M^{-1}.

Using the same method, the K_a value of PTX toward HSA was extrapolated to be 2.3×10^4 M^{-1}.

Determination of drug encapsulation efficiency in NPs

The drug encapsulation efficiency (EE) of DM1 in albumin-assembled NPs was determined by analytical HPLC. Briefly, 4.5 μL of DM1 (5 mg/mL in DMSO) and 1 mL of HSA solution (10 mg/mL in DI water), namely, a molar ratio of HSA to DM1 is 5:1, were mixed, and HSA NPs were prepared according to the above protocol. The NPs were precipitated using ultracentrifuge at 50,000 *g* for 45 min, and meanwhile, the unencapsulated DM1 in the supernatant were recovered. The DM1 molecules in supernatant were extracted with excess acetonitrile (H_2O/CH_3CN, v/v, 1/2) and analyzed by HPLC. Comparing the amount of un-encapsulated DM1 with that of initially added total DM1 from HPLC data (Fig. S5), we thus obtained the EE value. EE (%) = (1-weight of drugs in supernatant/weight of drugs fed initially) × 100. For HPLC analysis, YMC-Pack ODS-A analytical column (5 μm, 250 × 4.6 mm) was used. The mobile phase was a gradient of acetonitrile (solvent A) and water (solvent B) at a flow rate of 1.0 mL/min. The linear gradient was 20% A: 80% B to 100%A: 0% B within 20 min. UV detection was monitored at 220 nm.

Dynamic light scattering (DLS)

The as-prepared empty HSA nanoparticles and drug-loaded HSA nanoparticles was dispersed in DI water at a concentration of 0.2 mg/mL. The size distribution and zeta potential were measured using a Malvern Nano-ZS90 instrument (Malvern, UK) at 25°C.

Transmission Electron Microscopy (TEM)

The samples containing HSA NPs were placed onto a 300-mesh cooper grid for 2 min. The solution was then removed with a filter paper and air-dried. The samples were stained with 2wt% aqueous uranyl acetate and observed on TECNAL 10 (Philips).

Stability of maytamins in aqueous solution

The hydrodynamic diameters D_H of empty HSA NPs and DM1-loaded maytamins that were prepared at different molar ratios of HSA to DM1 were analyzed by DLS in phosphate buffered saline (PBS, pH 7.4).

Drug release kinetics

Cy3-labeled DM1 (conjugate **2**)-loaded HSA NPs were prepared according to the previous protocol. The molar ratios of HSA to **2** were fed at 5:1 and 1:2. The solutions containing **2**-HSA NPs were dialyzed against PBS (pH 7.4) using dialysis tubes (Spectrum, molecular weight cut off 14 kDa) and continuously stirred in an orbital shaking bath at 37°C. The solution (DMSO/H_2O, 1:9, v/v) containing free Cy3-DM1 conjugate **2** was included as a control. At pre-determined time intervals, the release media were collected and equal volumes of fresh media were added. The amounts of released **2** in the samples were determined by a UV-vis spectrophotometer (SHIMADZU, UV-2700) at a wavelength of 550 nm. To calculate the concentration of released drugs, the calibration curve as follows was used. The molar extinction coefficient (ε) was extrapolated to be 16,1407 $L \cdot mol^{-1} \cdot cm^{-1}$, which is consistent with the value reported by Lumiprobe (Florida, USA).

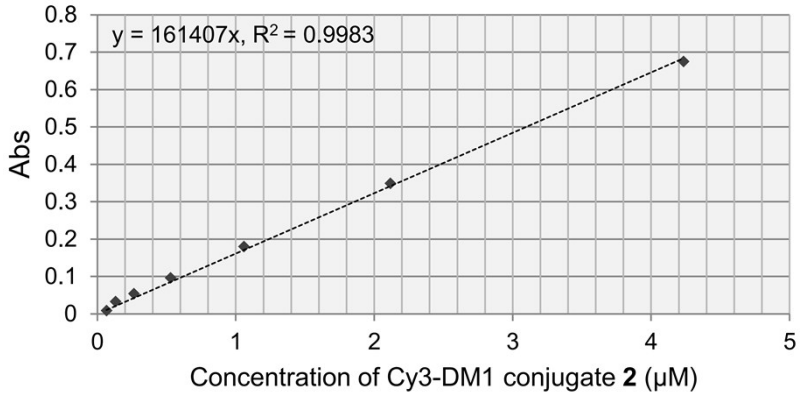


Figure S17 The calibration curve for Cy3-labeled DM1 compound **2**.

Cell culture

Human breast carcinoma Bcap37 cells and colon carcinoma LoVo cells were maintained in RPMI-1640 media. All media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were incubated at 37°C in a 5% CO_2 atmosphere.

***In vitro* MTT assay**

The *in vitro* cytotoxicity of various drugs was determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay in both human cancer Bcap37 and LoVo cell lines. Briefly, cells were evenly plated into 96-well plates at a density of 5000 cells per well and allowed to grow for 24 h. The cells were then added with empty HSA NPs, free DM1 and maytamins, and further incubated for 72 h at 37°C. Subsequently, 30 μL of MTT solution (5 mg/mL) was added into each well, and the plates were incubated for additional 4 h, allowing viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. Finally, the medium was removed, 100 μL of DMSO was added to dissolve the formazan crystals. The absorbance in each individual well was determined at 490 nm using a microplate reader (Multiskan FC, Thermo Scientific). The relative percentage of the untreated cells was used to represent 100% cell viability, and the concentrations of various drugs required to inhibit cell growth by 50% (IC_{50}) were extrapolated from the dose-response curves.

***In vitro* cell morphology changes**

Cell morphology changes upon drug treatment were observed in Bcap37 cells. Cells were plated into a 12-well plate at a density of 8×10^4 cells per well and allowed to grow overnight. Fresh culture media were replaced and cells were incubated with free DM1 (25 nM) and maytamins (25 nm with respect to DM1) for 24 h, 48 h and 72 h. The untreated cells were used as control. The cells were photographed under optical microscope (OLYMPUS, 1X71, Japan) at the time points of 0 h, 24 h, 48 h and 72 h.

***In vitro* cell cycle assay upon treating with maytamins**

In vitro cell cycle analysis was performed in Bcap37 cells. Briefly, cells were plated into a 6-well plate at a density of 2×10^5 cells per well and allowed to grow overnight. Fresh culture media were replaced and cells were incubated with free DM1 (25 nM) and

maytamins (25 nM with respect to DM1) for 24 h and 48 h. The untreated cells were used as control. At the end of incubation, cells were washed with cold PBS for 3 times and collected by centrifugation at 1000 rpm. The cells were fixed with 75% ethanol overnight at 4°C. The cell pellets were centrifuged (1500 rpm, 5 min, 4°C) and washed twice with PBS. For cell cycle analysis, propidium iodide (PI) detection kit (Sigma-Aldrich) was used according to the manufacturer's protocol. Briefly, the cells were suspended in 500 μL of PI working buffer and incubated for 15 min at room temperature in the dark. Finally, the cell samples were subjected to a flow cytometer (Beckman Coulter, FC500) to analyze the cell cycle phase distributions.

Immunofluorescence staining

Immunofluorescence staining was performed in Bcap37 cell line. Briefly, cells were plated into glass-bottom cell culture dish at a density of 4×10^4 cells per dish and allowed to grow overnight. Fresh culture media were replaced and cells were incubated with maytamins at 0, 25, and 50 nM DM1-equivalent concentrations for 12 h. At the end of incubation, cells were washed with cold PBS 3 times and fixed with 3.7% formaldehyde for 15 min at room temperature. Subsequently, the cells were treated with 0.5% Triton® X-100 permeabilization reagent for 20 min. The samples were then incubated with normal goat serum for 1 h and stained with anti-α-tubulin for 2 h at room temperature. The cells were washed with PBS 3 times and stained with Alexa Flour555 donkey anti-rabbit (1:2000) for 1h, protecting from light. After staining with 4' 6-diamidino-2-phenylindole (DAPI, 5 μg/mL) for 10 min, the cells were visualized using a fluorescence microscope (Nikon, ECLIPSE Ti-S, Japan).

***In vivo* drug toxicity**

The toxicity study using ICR mice was approved by The Animal Care and Use Committee of Zhejiang University. Healthy ICR mice (4-5 weeks old) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Science. They are housed under aseptic conditions and give autoclaved rodent diet and sterile water. The mice were randomized into 5 groups (n=10, five females and five males in each group) and intravenously injected with various drugs via the tail vein. Maytamins that were fabricated at a 5:1 molar ratio of HSA to DM1 were administered at doses of 1.25, 2.5, 5 mg/kg with respect to DM1. Free DM1 in ethanol/water (1/9, v/v) was injected at a

dose of 1.25 mg/kg. Saline was included as a control. Maytamins and free DM1 were administered every three day for 3 times. The body weight changes of mice were monitored. After 15 days, the mice were sacrificed by CO_2 inhalation.

References

S1 (a) S. Remillard, L. I. Rebhun, G. A. Howie, S. M. Kupchan, Antimitotic activity of the potent tumor inhibitor maytansine. Science, 1975, **189**, 1002–1005; (b) E. Oroudjev, M. Lopus, L. Wilson, C. Audette, C. Provenzano, H. Erickson, Y. Kovtun, R. Chari, M. A. Jordan, Maytansinoid-antibody conjugates induce mitotic arrest by suppressing microtubule dynamic instability. Mol. Cancer Therapeut., 2010, **9**, 2700-2713.