Supporting Information

Sub-10 nm Theranostic Unimolecular Micelles with High Tumor-Specific Accumulation, Retention and Inhibitory Effect

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Experimental section

Materials 10 × phosphate buffer saline (PBS) buffer with pH = 7.4 (ultrapure grade) is a commercial product of 1st BASE Singapore. MilliQ water (18.2 MQ) was used to prepare the buffer solution from the 10×PBS stock buffer. 1×PBS consists of NaCl (137 mM), KCl (2.7 mM), Na$_2$HPO$_4$ (10 mM) and KH$_2$PO$_4$ (1.8 mM). Chloroform-D (99%) was purchased from Cambridge Isotope Laboratories, Inc. All other chemicals and reagents were purchased from Aldrich or Merck and used as received unless other specified. Conjugated polymer PFTB-OH and CPT-BHD were synthesized according to our previous report.\textsuperscript{1,2}

Characterization The samples were dissolved with chloroform-d for $^1$H NMR measurements on a Bruker AV300 MHz NMR spectrometer. Molecular weight determinations for polymers were made using gel permeating chromatography (GPC) analyses (Agilent 1260, USA). The eluent was THF at a flow rate of 1.0 mL/min. A series of low polydispersity polystyrene standards were employed for the GPC calibration. Emission and excitation spectra of solutions were measured by a Horiba Fluolog 3 spectrofluorometer. UV/Vis spectra of the samples were measured on a SHIMADZU UV-2450 spectrophotometer. TEM measurements were performed with a TEM Carl Zeiss Libra 120 Plus at an acceleration voltage of 120 kV. A 5 μL droplet of diluted samples was directly dropped onto a copper grid (300 mesh) coated with a carbon film, followed by drying at room temperature. The size distribution of resulting nanoparticles was determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90°.
**Scheme S1** Synthetic route to monomer CPTA.

**Synthesis of Monomer CPTA CPT-BHD** (1.89 mmol, 1 g) and triethyl amine (TEA) (2.83 mmol, 0.39 mL) were mixed in a round bottom flask. THF (50 mL) was added to the flask via a syringe after purging with N\(_2\) gas, followed by cooling to 0 °C. Acryloyl chloride (2.27 mmol, 0.242 g) was then added via a micro syringe drop wise and the temperature of the flask was slowly raised to room temperature. After reaction for 24 h, the solution was filtered and evaporated the solvent. The solid was dissolved in 50 mL of DCM and washed by water (50 mL×3). The organic layer was dried by anhydrous MgSO\(_4\). After filtration, the mixture was concentrated via rotary evaporation and purified via column chromatography (silica gel) using hexane/EA (1/1 by vol) as eluent. The compound CPTA was obtained as white powder (0.35 g, yield: 31.8 %). The \(^1\)H-NMR spectrum of CPTA is shown in Figure S1.

**Synthesis of PFTB-CTA as a Macromolecular RAFT Agent** PFTB-OH (120 mg), 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (137.45 mg, 0.492 mmol) and 4-(Dimethylamino)pyridine (DMAP) (3 mg, 0.0246 mmol) were put in a two-neck round bottom flask. CH\(_2\)Cl\(_2\) (30 mL) was added to the flask via a syringe under the N\(_2\) atmosphere, followed by cooling to 0 °C. Dicyclohexylcarbodiimide (DCC) (0.344 mL, 1.5 mmol) was then dissolved in 5 mL of CH\(_2\)Cl\(_2\) and added via a micro syringe. The temperature of the flask was slowly
raised to room temperature. The reaction was continued for 24 h under stirring. The solution was filtered and precipitated in cold methanol to purify the product. The RAFT macroinitiator was then dried in vacuum at 50 °C to afford 123 mg of dark red powder. ¹H NMR (300 MHz, CDCl₃, δ ppm): 8.20-8.05 (m, 2H), 7.99-7.68 (m, 4H), 7.65-7.32 (m, 7H), 4.21-3.67 (m, 4H), 2.98-2.75 (br, 4H), 2.73-2.35 (m, 2.4H), 2.20-1.97 (m, 4H), 1.96-1.83 (br, 1.8H), 1.84-0.52 (m, 54H). GPC: \(M_n = 9,800, M_w/M_n = 2.65\).

**Synthesis of Polymer Bottlebrushes PFTB-\(g\)-(POEGMA-\(co\)-PCPTA)** Take UMPD1 as an example, PFTB-CTA (20 mg, molar of CTA = 0.031 mmol, 1eq.), olig(ethylene glycol) methyl ether methacrylate (OEGMA) (232.5 mg, 0.465 mmol, 15 eq.) and CPTA monomer (54.2 mg, 0.093 mmol, 3eq.) were dissolved in 1 mL of THF in a Schlenk tube. As followed, AIBN THF solution (1 mL × 0.01 mol/L, 0.33 eq.) was added to the mixture. After three freeze-and-thaw cycles for removal of oxygen, the tube was then placed in an oil bath thermostated at 70 °C for 48 h to allow RAFT. A solution of the crude product was precipitated in cold diethyl ether for three times. The final polymer brush UMPD1 was then dried in vacuum at 35 °C and obtained as a sticky fluid (272 mg). **UMWD, UMPD2, UMPD3 and UMPD4** were synthesized by the similar protocol and the detailed amount of monomer, initiator and AIBN were shown in Table 1. **UMPD1** for representative: ¹H NMR (300 MHz, CDCl₃, δ ppm): 8.57-7.34 (m, 12H), 5.77-5.21 (m, 8H), 4.42-4.27 (m, 380H), 3.48-3.29 (m, 32H), 3.05-2.83 (m, 8H), 2.4-0.5 (m, 88H). GPC: \(M_n = 48,700, M_w/M_n = 2.00\).

**Preparation of Unimolecular Micelles in Aqueous Medium** 1 mL of THF was used to dissolve the UMPDs (5 mg). After that, 5 mL of water or PBS buffer was directly added to
dilute the solutions. After the exposure in open air for the evaporation of THF for overnight the solutions with a concentration of 1 mg/mL were obtained and diluted to the desired concentration for further experiment.

**In Vitro Drug Release of UMPDs** As described above, the aqueous dispersions of UMPDs was obtained. The dispersion (1 mg/mL) was placed in 10 mM PBS buffer at pH 7.4 (0 mM DTT, 2 µM DTT, 0.1 mM DTT, 1 mM DTT or 5 mM DTT) and transferred to a dialysis tube (molecular weight cutoff: 3,500 Da) immersed in the same buffered media at 37 °C respectively. After varying time intervals, aliquots of the external medium were withdrawn and replaced with the same volume of fresh buffer solution. The fluorescence spectroscopy was employed to assay the CPT content to determine the CPT release rate based on a calibration curve with an excitation wavelength at 365 nm. The loading content (LC %) of CPT was calculated by the following equations:

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LC(\%) = \frac{\text{Molecular weight of CPT in UMPDs}}{\text{Molecular weight of UMPDs}} \times 100\%
\]

**Cell Culture** HepG2 human liver cancer cell, HeLa immortal cancer cells and MCF-7 breast cancer cells were cultured in folate-free Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified environment containing 5% CO₂. Before experiments, the cells were precultured until confluence was reached.

**In Vitro Cellular Uptake** HepG2, HeLa and MCF-7 cells were seeded in 12-wells plates at 37 °C (Costar, IL, USA). After 80% confluence, the medium was removed and the adherent cells were washed twice with 1× PBS buffer. The free CPT (10 µg/mL) and UMPDs (concentration
of CPT ~ 10 μg/mL) in DMEM medium were then added to different chambers, respectively. After incubation for 2h, 6h and even 11 h, the cells were washed five times with 1× PBS buffer and then fixed with 75% ethanol for 10 min. After washing it by 1× PBS buffer for three times, UMPD stained cells were directly recorded by a confocal laser scanning microscopy (LSM 710, Carl Zeiss, Germany). The lasers of 405, 514 nm were used to excite CPT and PFTB, respectively. The emission signals were collected by using a band pass filter combination 410-546, 546-735 nm for imaging in two individual channels. For free CPT stained cells, they were further blocked for 30 min in 1× PBS containing 1% (wt/vol) BSA. Then Alexa Fluor® 633 phalloidin diluted 20 times according to the protocol in 1× PBS was added to stain filamentous actin (F-actin) cytoskeleton for 1 h at room temperature. The cells was further washed by 1× PBS buffer and ready for characterization by CLSM (Objective: LD Plan-Neofluar 20x/0.4 Korr M27). Another laser of 633 nm was used to excite Alexa Fluor® 633 phalloidin. The corresponding fluorescence emission was recorded using a band-pass filter combination including 410-546 nm, 546-735 nm and 638-735 nm for imaging of CPT, PFTB and Alexa Fluor® 633 phalloidin, respectively.

**Cytotoxicity study** The cytotoxicity of UMPDs against HepG2 and HeLa cancer cells was evaluated by PrestoBlue (PB) assay. Briefly, HepG2 and HeLa cells were seeded in 96-well plates (Costar, IL, USA) at a density of 1×10⁴ cells mL⁻¹. After 24 h incubation, the cells were exposed to a series of doses of UMPDs at 37 °C. After the designated time intervals, the wells were washed twice with 1× PBS buffer and 100 μL of freshly prepared PB solution in culture medium was added into each well. The PB medium solution was carefully removed after 1 h incubation in the incubator. The absorbance of PB at 570 nm and 600 nm was monitored by
the microplate reader (Genios Tecan). Cell viability was expressed as the ratio of the percent PB reduction of the cells incubated with UMPDs suspension to that of the cells incubated with culture medium only.

**Tumor mouse model** The care and use of laboratory animals were performed according to the approved protocols of the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore. All animal experiments were performed in compliance with the Guidelines established by the Institutional Animal Care and Use Committee (IACUC), SingHealth. To establish tumor models in 4-week-old female nude mice, $1 \times 10^6$ HepG2 cancer cells in 100 μL of supplemented MEM (10% fetal bovine serum, 1% pen/strep (100 U/ml penicillin and 100 mg/mL streptomycin) were injected subcutaneously in the right flank region of the mouse. Tumors were allowed to grow to ~150 mm$^3$ before being used for in vivo imaging experiments and chemotherapy treatment.

**In vivo and ex vivo optical imaging** In the experiments of long-term monitoring of biodistribution, after the nude mice were anesthetized using 2% isoflurane in oxygen, UMPD1 (25 μg/mL) (n = 2) was systematically injected through the tail vein using a microsyringe. Fluorescence whole animal imaging was performed using a IVIS® Spectrum CT whole animal imaging system. The fluorescence distribution was monitored at 0, 0.5, 24, 48, 72, 96, 120, 144, 168 h after UMPD1 administration using the in vivo imaging system with appropriate wavelength ($\lambda_{ex} = 535$ nm, $\lambda_{em} = 660$ nm). Mice were sacrificed by cervical dislocation under deep isoflurane anesthesia at 72 h post injection. The liver, tumor, stomach, spleen, kidney, heart and intestine were harvested for ex vivo fluorescence imaging to estimate the tissue
distribution of the NPs. The in vivo long-term monitoring based UMPD4 was conducted by the similar procedure at different time points.

**In vivo anticancer activity** The HepG2 tumor-bearing nude mice were randomly divided into three groups. Mice were intravenously injected with UMPD1 (CPT dose of 4 μg/g), UMWD (polymer dose of 27 μg/g) and phosphate buffer solution (1 × PBS dose of 200 μL) via tail vein every 3 days over 18 days (n = 3 per group). Each mouse of different group was earmarked and followed individually throughout the whole experiments. Fluorescence whole animal imaging was also conducted every 3 days to track the variation of biodistribution and fluorescence intensity (λex = 535 nm, λem = 660 nm). The length and width of the tumor and the body weight of mice were measured before every injection by the end of experiment. Tumor volume (V) was calculated using the formula: V (mm³) = 1/2 × length (mm) × width (mm)². After 18 days postinjection, the mice were sacrificed, and tumors were separated, weighted and photographed.
Figure S1 $^1$H NMR (CDCl$_3$, 300 MHz) spectrum of monomer CPTA.
Figure S2 (a) $^1$H NMR (CDCl$_3$, 300 MHz) spectra of (a) PFTB$_9$-OH, (b) (PFTB-g-CTA$_{1.2}$)$_9$ and (c) UMPD1. GPC traces of UMPD1 and its precursor (macroinitiator) using RI detector (d) and UV-vis detector (e) (wavelength: 500 nm).
**Figure S3** GPC traces of conjugated polymer bottlebrushes PFTB-\(g\)-(PCPTA-co-POEGMA)

(a) **UMP2** and (b) **UMP3** using RI and UV-vis detectors (wavelength: 500 nm).
Figure S4 DLS results of (a) UMWD, (b) UMPD2, (c) UMPD3, (d) UMPD4 in aqueous media and (e) UMPD1, (f) UMPD2, (g) UMPD3 in THF.
Figure S5 DLS results of UMPD after stored over 6 months. UMPD1 by number distribution: (a) 0.01 mg/mL, (b) 0.1 mg/mL, (c) 1 mg/mL; UMPD1 by volume distribution: (d) 0.01 mg/mL, (e) 0.1 mg/mL, (f) 1 mg/mL; UMPD2 by number distribution: (g) 0.01 mg/mL, (h) 0.1 mg/mL, (i) 1 mg/mL.
Figure S6 TEM image of air-dried **UMPD2** (a) and **UMPD3** (b).
Figure S7 In vitro CPT release profiles of UMPD1 in PBS buffer upon treating with DTT of varying concentrations: (■) 0.1 mM DTT; (●) 1 mM DTT.
**Figure S8** DLS histogram of UMPD1 in water recorded in the presence of DTT (10 mM)
Figure S9 UV-vis absorption and FL emission ($\lambda_{ex} = 365$ nm) spectra of (a) UMPD2 and (b) UMPD3 in water. FL emission spectra and excitation spectra of (c) (d) UMPD2 and (e) (f) UMPD3 recorded in the presence and absence of DTT (10 mM).
Figure S10 CLSM images of HepG2 cells at lower magnification after incubation with aqueous dispersion of free CPT at a CPT equivalent dosage of 10 µg/mL for 2 h and aqueous dispersion of UMPD1 for 2 h, 6 h, and 11 h.
**Figure S11** CLSM images of HeLa cells after incubation with aqueous dispersion of free CPT at a CPT equivalent dosage of 10 $\mu$g/mL for 2 h and aqueous dispersion of UMPD1 for 2 h, 6 h.
Figure S12 CLSM images of MCF-7 cells after incubation with aqueous dispersion of free CPT at a CPT equivalent dosage of 10 μg/mL for 2 h and aqueous dispersion of UMPD1 for 2 h, 6 h.
Figure S13. The distribution profiles of **UMPD1** (a) and **UMPD4** (b) in excised organs based on the total fluorescence intensity at different time points postinjection. Data are given as mean ± standard error (n=2).
REFERENCES