Supplementary information:

Drug-loaded pseudo block copolymer micelles with multi-armed star polymer as the micellar exterior

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Materials and Methods

1. Materials

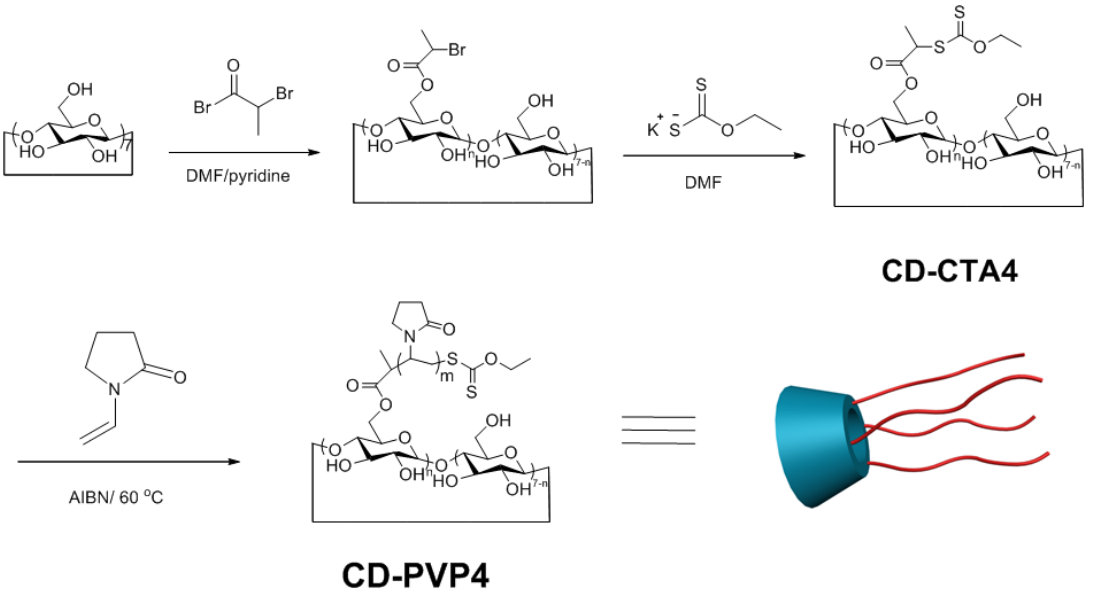
β-cyclodextrin (β-CD), N-vinylpyrrolidone (NVP), 2-bromopropionic acid, potassium O-ethyl xanthate, triphenylphosphine, iodine, 1-adamantylamine, stannous octotate, azodiisobutyronitrile (AIBN), 4-dimethylaminopyridine (DMAP), diisopropylcarbodiimide (DIC), copper(II) sulfate pentahydrate, sodium ascorbate, rhodamine B, bovine serum albumin (BSA), Nile red and bis(2-phenylbenzothiazolato)-(acetylacetonate)iridium(III) ($Ir(btb)_2(acac)$) were purchased from Sigma-Aldrich. 2-bromopropionyl bromide and ε-caprolactone (ε-CL) were purchased from TCI. Sodium azide and propargyl alcohol were purchased from Shanghai Chemical Reagent Co., China. All the other solvents and reagents were of analytical grade and used without further purification. N-vinylpyrrolidone and ε-caprolactone were dehydrated by CaH_2 and distilled under vacuum before use. Human non-small lung cancer cell line, A549, and murine hepatic cell line, H22, were purchased from Shanghai Institute of Cell Biology (Shanghai, China). Paclitaxel (PTX)-resistant human ovarian cell line, A2780/T, was purchased from KeyGEN BioTECH Co. (Nanjing, China). Male ICR mice were purchased from Animal Center of

Drum Tower Hospital (Nanjing, China). Cabazitaxel was kindly provided by Biocompounds Pharmaceutical Inc. (Shanghai China).

2. Synthesis of adamantane-terminated poly(ε-caprolactone) (ada-PCL)

Ada-PCL was prepared by ring opening polymerization of ε-CL (2.26 g, 19.8 mmol) using 1-adamantylamine (30 mg, 0.198 mmol) as the initiator and stannous octotate (0.05% w/w) as the catalyst according to a well-established method.[1] Briefly, 1-adamantylamine (30 mg, 0.198 mmol) was dissolved in freshly distilled ε-CL (2.26 g, 19.8 mmol) followed by adding stannous octotate (0.05% w/w) in a 50 mL schlenk flask. The flask was sealed under vacuum and then immersed into a 120 °C oil bath to start the polymerization. After 48 hour the reaction was stopped by cooling to room temperature, and 10 mL of dichloromethane (DCM) was added to dilute the product. The obtained solution was precipitated into excess cold diethyl ether to give the white precipitate as the product. The precipitate was collected by filtration and purified by washing with diethyl ether and methanol several times. The final product was dried under vacuum at room temperature. Yield: 2.1 g, 91.7%.

3. Synthesis of 4 arm PVPs grafted from β-cyclodextin (CD-PVP4)



Scheme S1 Synthesis route of 4 PVP chains modified β-CD, CD-PVP4

3.1 Synthesis of 4-[6-(2'-bromopropionic ester)]-β-cyclodextin (CD-Br4)

β-cyclodextin (1g, 0.88 mmol) and pyridine (0.55 mL, 6.8 mmol) were dissolved in 20 mL of anhydrous N, N-dimethylformamide (DMF) and then cooled to 0 ^{o}C, a solution of 2-bromopropionyl bromide (0.55 mL, 5.28 mmol) in anhydrous DMF (5 mL) was added dropwise into β-cyclodextrin solution with stirring for 0.5 h under 0 ^{o}C. After addition, the reaction temperature was allowed to rise to room temperature naturally and the solution was maintained stirring at room temperature for another 16 h. After which the solution was precipitated into excess diethyl ether, the obtained white powder was collected, washed with diethyl ether several times and then dried in vacuum oven at room temperature. The obtained crude product was then suspended in 30 mL of water and stirred at room temperature for 12 h. After that the remaining solid was collected by filtration and then suspended in 20 mL acetone with stirring for another 12 h. After which the solid was collected by centrifugation and washed with acetone (3 × 10 mL). The obtained white solid was dried in vacuum oven at room temperature to give the product CD-Br4. Yield: 0.64g, 30.5%.^{1}H NMR (DMSO-d_6, 400 MHz, δ): 1.70 (s, 10.5H, CH_3 of -OCO-CH(CH_3)Br), 4.85 (s, 7H, -O-CH-O-), 3.00-6.00 (m, 70H, protons of β-CD and CH of –OCO-CH(CH_3)Br). ^{13}C NMR (DMSO-d_6, 100 MHz, δ): 21.99, 59.85, 65.19, 69.41, 72.50, 73.32, 82.13, 101.89, 102.87, 170.24.

3.2 Synthesis of 4-[6-(2'-(O-ethyl xanthate) propionic ester)]-β-cyclodextin macroCTA (CD-CTA4)

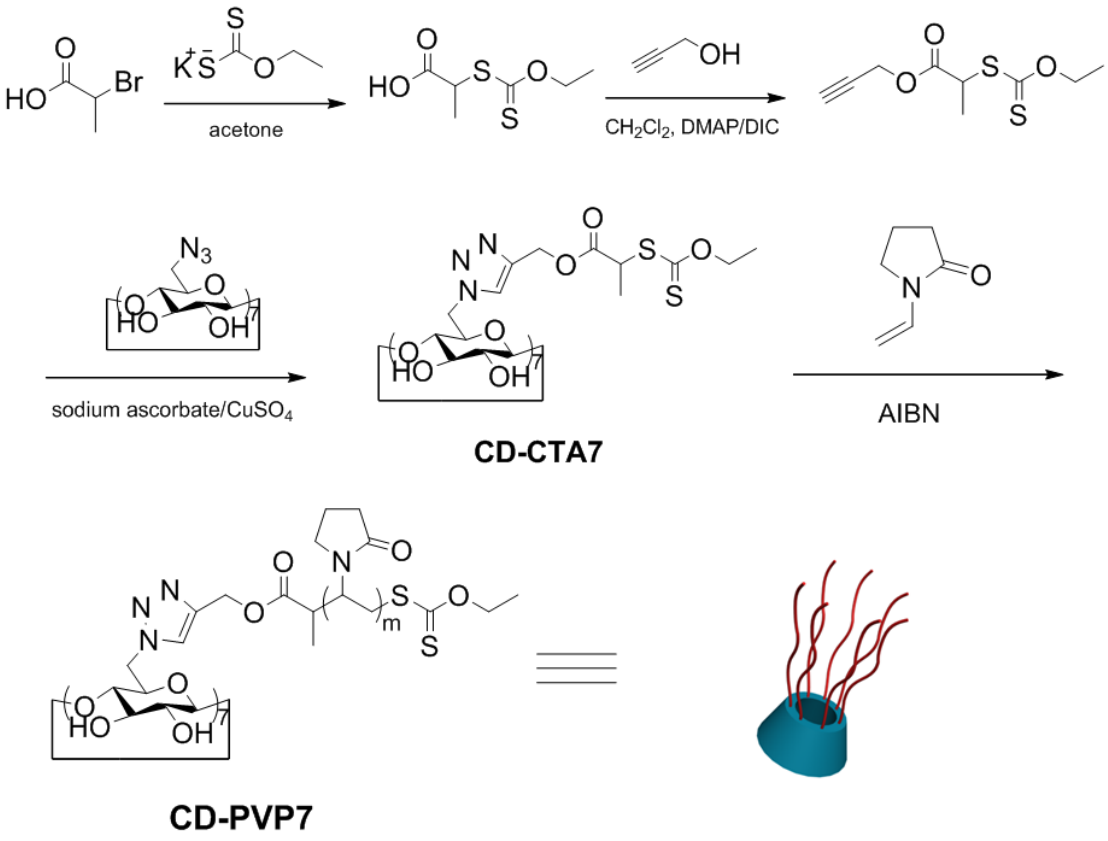
CD-Br4 (0.5g, 0.32 mmol) and pyridine (0.5 mL, 6.2 mmol) were dissolved in 10 mL of anhydrous DMF followed by addition of potassium O-ethyl xanthate (0.23 g, 1.46 mmol). The solution was stirred at room temperature for 16 h. Then the solution was precipitated *via* aforementioned method. The obtained pale yellow solid was then washed with water (3 × 10 mL) and dried in vacuum to give CD-CTA4. Yield: 0.42g, 57.5%.^{1}H NMR (DMSO-d_6, 400 MHz, δ): 1.08-1.60 (m, 21H, CH_3 of -OCO-CH(CH_3)S- and –OCH_2CH_3), 4.84 (s, 7H, -O-CH-O-), 3.00-6.00 (m, 77H, protons

of β-CD and CH of –OCO-CH(CH_3)S and CH_2 of –OCH_2CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz, δ): 13.75, 17.00, 46.57, 59.88, 64.77, 69.41, 71.13, 72.69, 73.36, 82.33, 102.96, 170.82, 211.70. Elemental analysis for C63H98S7O42·2H2O (DS=3.5), calculated C, 42.33; H, 5.71; determined C, 42.03; H, 6.12.

3.3 Synthesis of CD-PVP4

CD-CTA4 (0.25 g, 0.15 mmol) was dissolved in 3 mL of freshly distilled NVP followed by adding AIBN (2.5 mg, 0.015 mmol). The solution was degassed *via* three freeze-pump-thaw circles then immersed into a 60 °C oil bath to start the reaction. The reaction was stopped at 72 h by cooling to room temperature and exposing to the air. The final solution was diluted with DCM and precipitated with excess diethyl ether. The obtained white solid was collected by filtration and washed with diethyl ether several times then dried under vacuum at room temperature. Yield: 2.5 g, 76.9%.

4. Synthesis of 7 arm PVPs grafted from β-cyclodextin (CD-PVP7)



Scheme S2 Synthetic route of 7 PVP chains modified β-CD, CD-PVP7

4.1 Synthesis of propargyl 2-(O-ethyl xanthate) propionic ester (alkynyl-CTA)

Alkynyl-CTA was synthesized based on previous literature with modification.[2] 2-bromopropionic acid (2 mL, 22.2 mmol) was added into 35 mL of acetone. Subsequently, potassium O-ethyl xanthate (5.3 g, 33.1 mmol) was added to 2-bromopropionic acid solution by small portion with stirring. The mixture was stirred at room temperature for 24 h. After which the mixture was filtered to remove the precipitation, acetone was then removed by rotary evaporation. The residue was diluted with chloroform (40 mL) and then washed with water (30 mL) for 3 times. The solution was dried over $MgSO_4$ overnight and then evaporated to give 2-propionic acid O-ethyl xanthate as yellow oil. Yield: 3.3 g, 37.9%. ^{1}H NMR ($CDCl_3$, 400 MHz, δ): 1.42 (t, 3H), 1.60 (d, 3H), 4.42 (d, 1H), 4.64 (tt, 2H), 10.96 (s, 1H).

Propargyl alcohol (2 mL, 34.6 mmol), 2-propionic acid O-ethyl xanthate (6 g, 30.9 mmol) and DMAP (400 mg, 3 mmol) were dissolved in 50 mL of DCM and immersed into an ice bath. DIC (4.46 mL, 30.9 mmol) was then added into the

solution under stirring. After which the reaction was maintained at 0 °C for 30 min and allowed to rise to room temperature naturally, then the reaction was carried on for another 48 h. The solution was filtered to remove the by-product urea and then concentrated by rotary evaporation. The residue was purified by column chromatography with an eluant of n-hexane/ ethyl acetate (3:1) to give the product alkynyl-CTA. Yield: 5.72 g, 72%.^{1}H NMR ($CDCl_3$, 400 MHz, δ): 1.42 (t, 3H), 1.59 (d, 3H), 2.50 (t, 1H), 4.41 (d, 1H), 4.63 (q, 2H), 4.74 (d, 2H).

4.2 Synthesis of 7 alkynyl-CTA groups modified β-cyclodextin macroCTA (CD-CTA7)

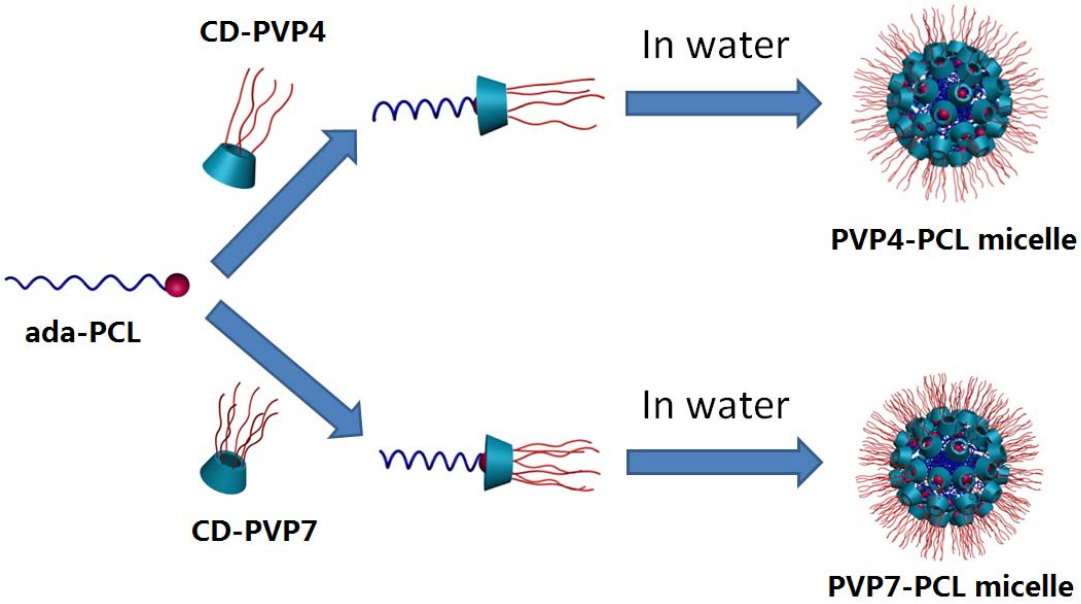
Heptakis(6-Deoxy-6-Azido)-β-cyclodextrin (CD-$(N_3)_7$) was synthesized according to previous literature.[3] CD-$(N_3)_7$ (100 mg, 0.076 mmol) and alkynyl-CTA (136 mg, 0.588 mmol) were dissolved in 20 mL of anhydrous DMF. Copper (II) sulfate pentahydrate (133 mg, 0.532 mmol) and sodium ascorbate (211 mg, 1.06 mmol) were then added to the solution. The resulting mixture was flushed with argon for 10 min. After which the mixture was immersed into a 60 °C oil bath and stirred for 24 h. The mixture was filtered and the resulting solution was precipitated into excess diethyl ether to give the crude product. Subsequently, the product was washed with diethyl ether, methanol and water to give the final product CD-CTA7. Yield: 212 mg, 89.7%.^{1}H NMR (DMSO-d_6, 400 MHz, δ): 1.11-1.55 (m, 42H, CH_3 of -OCO-CH(CH_3)S- and –OCH_2CH_3), 3.00-6.28 (m, 84H, protons of β-CD and CH of –OCO-CH(CH_3)S and CH_2 of –OCH_2CH_3), 7.98 (s, 7H, -N-CH-C-). ^{13}C NMR (DMSO-d_6, 100 MHz, δ): 13.76, 16.86, 46.59, 49.95, 58.63, 70.52, 71.92, 72.25, 72.78, 83.17, 102.16, 126.86, 141.47, 170.70, 211.56. Elemental analysis for $C_{105}H_{147}N_{21}O_{49}S_{14}\cdot 2H_2O$, calculated C, 42.44; H, 5.08; N, 9.90; determined C, 41.76; H, 5.15; N, 9.77.

4.3 Synthesis of CD-PVP7

The polymerization procedure of CD-PVP7 was similar to CD-PVP4. CD-CTA7 (0.3g, 0.1 mmol) was dissolved in 5 mL of freshly distilled NVP followed by addition of AIBN (1.7 mg, 0.01mmol). The solution was degassed *via* three freeze-pump-thaw

circles and then immersed into a 60 °C oil bath to begin the reaction. The reaction was stopped at 120 h by cooling to room temperature and exposing to the air. The viscous solution was diluted with DCM (10 mL) and precipitated with excess diethyl ether. The obtained white solid was collected by filtration and washed with diethyl ether several times then dried under vacuum at room temperature. Yield: 4.1 g, 77.3%.

5. Preparation of PVP-PCL pseudo block copolymer micelles



Scheme S3 Constructions of PVP4-PCL micelle and PVP7-PCL micelle

5.1 Preparation of 4 armed PVP and linear PCL pseudo block copolymer micelles (PVP4-PCL micelles)

Ada-PCL (10 mg, 0.001 mmol) and CD-PVP4 (19 mg, 0.001 mmol) were dissolved into 1 mL of DMF. To this solution was added 3 mL of 60 °C water rapidly. The solution turned into bluish immediately which indicated the formation of nanoparticles. After cooling to room temperature, the solution was dialyzed against water for 24 h to remove DMF. After that the solution was filtered to remove aggregates to give the final PVP4-PCL miclles.

5.2 Preparation of 7 armed PVP and linear PCL pseudo block copolymer micelles (PVP7-PCL micelles)

The procedure of preparation of PVP7-PCL micelles was almost the same as preparation of PVP4-PCL micelles. Briefly, ada-PCL (10 mg, 0.001 mmol) and CD-PVP7 (24 mg, 0.001 mmol) were dissolved into 1 mL of DMF. To this solution was added 3 mL of 60 ^{o}C water rapidly. The solution was dialyzed against water for 24 h and then filtered to give PVP7-PCL micelles.

6. Preparation of cabazitaxel (CBZ)-loaded PVP7-PCL micelles

The prepared PVP7-PCL micelles were used to prepare cabazitaxel-loaded micelles. CBZ (4 mg, 0.0048 mmol) was dissolved in 1 mL of DMF, PVP7-PCL micelle solution which was heated to 60 ^{o}C beforehand was added into cabazitaxel solution rapidly. The solution was dialyzed against water for 24 h and then filtered to give the CBZ-loaded PVP7-PCL micelles solution. The solution was then lyophilized for further use.

7. Characterization of PVP-PCL micelles

Hydrodynamic diameters and size distributions of PVP-PCL micelles were determined by dynamic light scattering (DLS) using a Brookhaven BI9000AT system (Brookhaven Instrument Co., USA). Each sample was adjusted to a proper concentration before measurement. Zeta potential of the nanoparticles was examined by Zetaplus (Brookhaven Instrument Co., USA). All the measurements were repeated three times and the results were chosen as the average of three runs.

The morphology and diameter of nanoparticles in dry state were obtained by transmission electron microscopy (TEM) (JEOL TEM-100, Japan). Samples were placed onto copper grill and air-dried at room temperature before observation.

8. Protein adsorption on nanoparticles

100 μL of micelles solution (4 mg/mL) was added into 900 μL of BSA solution (1 mg/mL), and the solution was incubated at 37 °C for 2 hour. After that the solutions were centrifuged (20,000 g, 30 min) and the supernatant was discarded. 1 mL of PBS (pH = 7.4, 10 mM) was added to wash the pellet three times. The pellet was then redispersed into 1 mL of PBS (pH = 7.4, 10 mM) and was then added into 96-well plate. Micro bicinchninic acid (BCA) protein assay was exploited to determine the amount of protein by using microplate reader at a wavelength of 562 nm.

9. Determination of drug loading content and encapsulation efficacy

The lyophilized CBZ-loaded PVP7-PCL micelles were dissolved in methanol, respectively. The solutions were sonicated for 15 min and then centrifuged at 1000 g for 5 min. The supernatant of solutions was collected for HPLC measurements.

HPLC measurements were achieved using a HC-C18 column (250×4.6 mm, 5 μm, C18, Agilent Technologies, Palo Alto, USA). The mobile phase was composed by double distilled water and acetonitrile (HPLC grade) with the ratio of 42/58. The flow rate was set as 1.0 mL/min, the wavelength of UV detector was 228 nm. The retention time of cabazitaxel is 8.2 min. The drug loading content (DLC) and encapsulation efficacy (EE) were calculated as follows:

$$DLC(\%) = \frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of the whole nanoparticles}} \times 100\% \qquad (1)$$

$$EE(\%) = \frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of the feeding drug}} \times 100\% \qquad (2)$$

10. *In vitro* release of cabazitaxel from micelles

1 mL of predetermined drug content micelles was placed into a dialysis bag (MWCO 12kDa). The dialysis bag was then immersed into 5 mL release medium (PBS, 0.01M, pH=7.4, containing 0.1% v/v Tween 80) and put in incubator at 37 °C. At predetermined time intervals, the release medium was withdrawn completely and

equivalent fresh release medium was added. The cabazitaxel content of collected release medium was determined by HPLC under the same condition described above.

11. *In vitro* cytotoxicity

In vitro cytotoxicity of CBZ-loaded micelles against two cell lines including A549 and PTX-resistant human ovarian A2780/T were assessed by MTT. A549 and A2780/T cells were seeded (5000 cells per well) in 96-well plate, respectively. Then A549 cells were exposed to a series of 200 μL of medium containing free cabazitaxel, empty PVP7-PCL micelles and CBZ-loaded PVP7-PCL micelles, respectively. A2780/T cells were exposed to paclitaxel, cabazitaxel, empty PVP7-PCL micelles and CBZ-loaded PVP7-PCL micelles, respectively. Each series was incubated for 48 h at 37 ^{o}C in a humidified atmosphere with 5% CO_2. After co-incubation, the culture medium was removed and then washed with PBS (0.01M, pH = 7.4) twice in each well, 20 μL of MTT dye (5 mg/mL) solution with 180 μL of fresh culture medium was added to each well. Additional 4 h was taken to incubate the cells, after which the medium was removed and 150 μL of DMSO was added into each well to dissolve the formazan crystals. The wavelength of microplate reader was set at 570 nm. The cell viability (%) was calculated as follows:

$$Cell\ Viability(\%) = \frac{\text{Abs(test cell)}}{\text{Abs(controlled cell)}} \times 100\% \qquad (3)$$

12. Cellular uptake of nanoparticles

Fluorescent dye-labeled micelles were prepared as follows: ada-PCL (100 mg, 0.01 mmol), DMAP (5 mg, 0.038 mmol) and rhodamine B (48 mg, 0.1 mmol) were dissolved in 5 mL of DCM. DIC (15 μL, 0.1 mmol) was then added into the solution. The reaction between the hydroxyl at end of ada-PCL and rhodamine B was carried out at room temperature for 24 h. After that, the solution was precipitated with

excess diethyl ether to obtain a red solid and then washed with diethyl ether and methanol to give the product ada-PCL-RB. The synthesized ada-PCL-RB was used to prepare rhodamine labeled CD-PVP7 micelles (RB-labeled PVP7-PCL micelles) with the method described above.

Subsequently, for cellular uptake, A549 and A2780/T cells were seeded in 6-well plate with the cell density of 2.5×10^5 cells per well, respectively. The cells were then incubated at 37 ^oC for 24 h in a humidified atmosphere with 5% CO2. 200 μL of RB-labeled PVP7-PCL micelles was added into the 6-well plate. After another 4 h of incubation at 37 ^oC, the cells were washed with PBS (0.01M, pH=7.4) three times in each well. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus of cells. The cells were then observed by confocal laser scanning microscopy (CLSM). Rhodamine B and DAPI excitations were achieved with a 543 nm HeNe laser and a 405 nm diode laser, respectively. The pin-hole diameter was set as 1 AU.

13. Cell uptake and penetration in multicellular tumor spheroids (MCTS)

Human neuroblastoma cell line SH-SY5Y was utilized to culture MCTS according to our previous work.[4] SH-SY5Y MCTS with diameter between 200 ~ 300 μm were harvested after 7 days of growth. For each experiment, about 20 spheroids were handpicked by a Pasteur pipette and then transferred to a 5 mL eppendorf tube. RB-labeled PVP7-PCL micelles were then added into the spheroids suspension and co-incubated at 37 ^oC for different time, respectively. At predetermined time point, the medium was removed and the spheroids were washed with PBS (pH = 7.4). The cellular uptake of micelles by spheroids was then observed by CLSM. For the penetration experiments, individual spheroid was observed at each 15 μm section from the top to equatorial plane by using CLSM. To determine the mean fluorescent intensity of each spheroid, fluorescent intensity of all interest regions was counted and normalized by area.

14. *In vivo* biodistribution of nanoparticles

Luminescent $Ir(btb)_2(acac)$ was used as the model drug to examine the biodistribution of micelles. Ir-loaded PVP7-PCL micelles were prepared using the same method which prepared CBZ-loaded PVP7-PCL micelles.

All animal studies were reviewed and performed under guidance of Animal Care and Use Committee, Nanjing University. $4\text{-}6\times10^6$ H22 cells were inoculated subcutaneously into the left flank of ICR mice (male, 6-7 weeks, 20-25 g). The mice were fed with food and water for 7 days. After that, 15 H22 tumor bearing ICR mice were injected *via* tail vein with Ir loaded micelles at an equivalent dose of 3 mg/kg body weight. Free $Ir(btb)_2(acac)$ which dissolved in saline was also injected in the same number of mice as the control. Another 3 mice without any injection were served as blank group. The mice were sacrificed at 1 h, 4 h, 8 h, 12 h and 24 h after i.v. administration. 3 mice were used at each time point respectively. Blood samples were collected *via* eye puncture and then centrifuged at 1000 g for 10 min to obtain plasma. Subsequently, other organs were collected carefully and then washed with water. All the collected organs including plasma were weighed and suspended in 3 mL of DCM with vigorously homogenize, the samples were then extracted at room temperature for 24 h. After that, the samples were filtered through 0.22 μm sized cellulose acetate filter membrane to remove the solid. The fluorescence of the filtrate was measured by fluorescence spectroscope (Shimadzu, RF-5301PC, Japan) with an excitation wavelength of 480 nm and emission wavelength of 613 nm. The average fluorescent intensity of each tissue from blank group was served as background. The final fluorescent intensity of each sample was obtained after subtracting the background.

15. *In vivo* antitumor efficacy of CBZ-loaded micelles

H22 tumor bearing mice were fed as described above. When the tumor volume reached about 50 mm^3 (designated as Day 1), 45 H22 tumor bearing mice were randomly divided into 5 groups (9 mice per group). 200 μL of saline, empty PVP7-PCL micelles, paclitaxel (10 mg/kg), cabazitaxel (10 mg/kg), CBZ-loaded PVP7-PCL

micelles (10 mg/kg cabazitaxel eq.) were administrated *via* tail vein, respectively. The tumor volume and body weight of mice were measured on alternate day. The volume of tumor was calculated as follows:

$$\text{Volume} = \frac{1}{2} D d^2 \qquad (4)$$

In the equation, D represents the maximum diameter of tumor while d represents the minimum diameter of tumor. Tumor growth inhibition (TGI) rate was calculated as follows:

$$\text{TGI} = (1 - \frac{\text{tumor volume of tested group}}{\text{tumor volume of saline group}}) \times 100\% \qquad (5)$$

16. Statistical analysis

Comparisons were performed by ANOVA analysis and Student's t-test. P value < 0.05 was accepted as statistically significance.

Supplementary Figures

Table S1. Molecular weight parameters of synthesized polymers.

Figure S1. ^{1}H NMR spectrum of ada-PCL.

Figure S2. ^{1}H NMR spectra of CD-PVP4 and CD-PVP7.

Table S1 Molecular weight parameters of synthesized polymers

Samples	M_n(^{1}H NMR)	M_n(GPC)	M_w(GPC)	M_w/M_n
Ada-PCL	11300	9800	11400	1.16
CD-PVP4	NA[a]	19000	27200	1.43
CD-PVP7	NA[a]	24300	36800	1.51

[a] The molecular weight of CD-PVP4 and CD-PVP7 cannot be calculated by ^{1}H NMR due to serious overlap of β-CD signals and PVP signals.

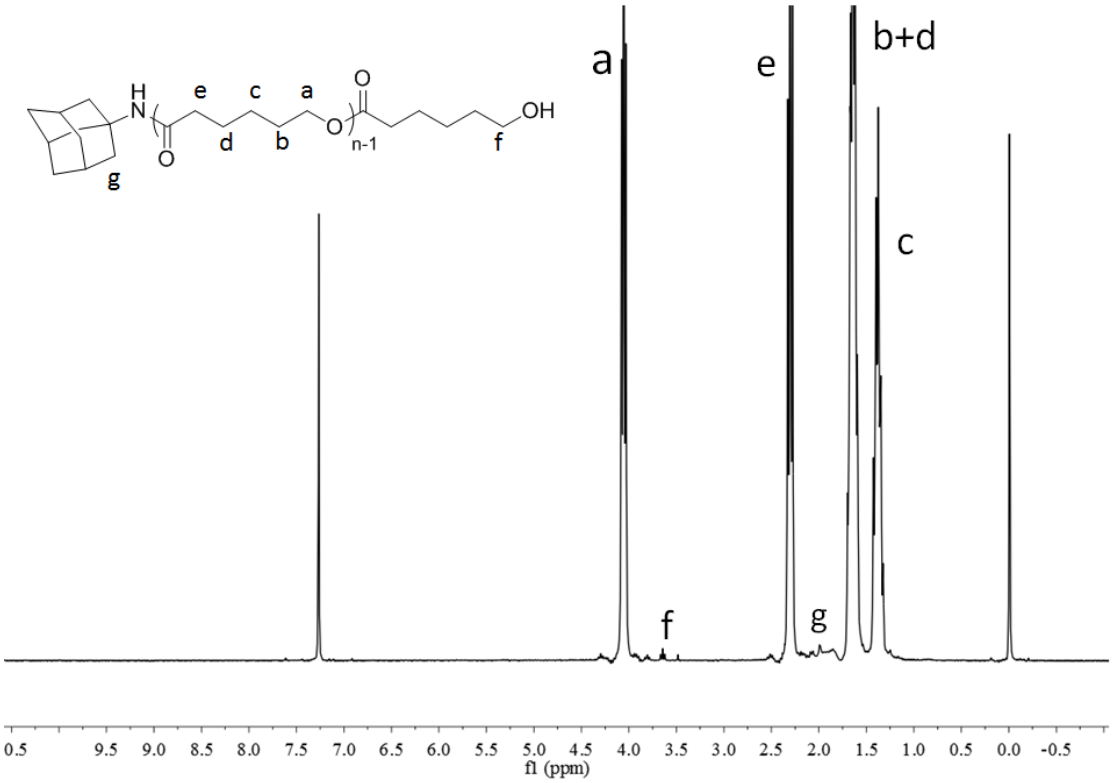


Figure S1 ^{1}H NMR spectrum recorded in $CDCl_3$ for ada-PCL

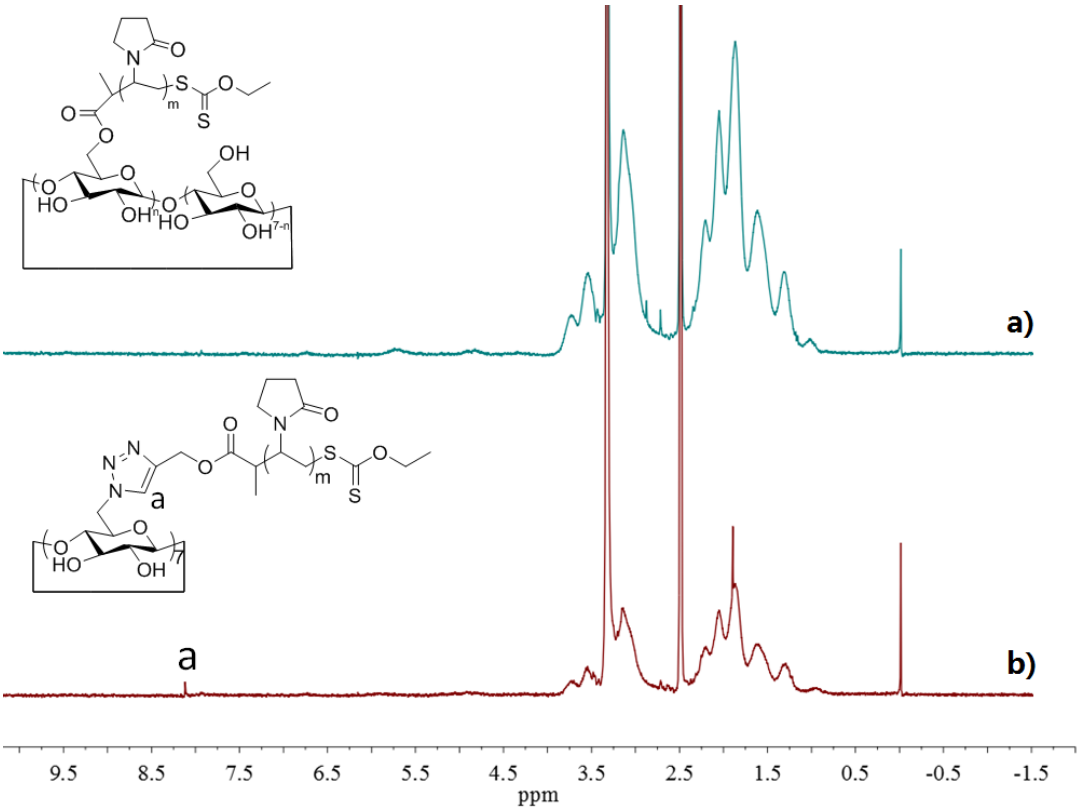


Figure S2 ^{1}H NMR spectra recorded in d_6-DMSO of (a) CD-PVP4 and (b) CD-PVP7.

References

1 L. Zhang, Y. Hu, X. Jiang, C. Yang, W. Lu and Y. Yang, *J. Controlled Release*, 2004, **96**, 135-148.

2 C. Xiao, D. Lu, S. Xu and L. Huang. *Starch/Starke*, 2011, **63**, 209–16.

3 Z. Ge, J. Xu, J. Hu, Y. Zhang and S. Liu. *Soft Matter*, 2009, **5**, 3932-3939.

4 X. Wang, X. Zhen, J. Wang, J. Zhang, W. Wu and X. Jiang. *Biomaterials*, 2013, **34**, 4667-4679.