Electronic Supplementary Material (ESI) for ChemComm.

Dual pH-responsive 5-aminolevulinic acid pseudopolyrotaxane prodrug micelles for enhanced photodynamic therapy

Hongxin Tong, Yin Wang, Huan Li, Qiao Jin*, Jian Ji*

MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China.
E-mail: jinqiao@zju.edu.cn, jijian@zju.edu.cn; Fax: +86-571-87953729; Tel: +86-571-87953729

Experimental section

Materials

α-Cyclodextrin (α-CD) was supplied by Aladdin Reagent Co., Ltd. (Shanghai, China) and dried for 48 h in a vacuum oven before use. Hydrazine hydrate aqueous solution (85%) was purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd. 4-Nitrophenyl chloroformate was provided by Energy Chemical Co., Ltd.(Shanghai, China). Monomethoxypoly(ethylene glycol) -RRRRRRHHHH (R6H4) was synthesized by Top-peptide Co., Ltd (Shanghai, China). 5-Aminolevulinic acid hydrochloride (ALA·HCl, 99%) was supplied by Dalian MeiLun Biotechnology Co., Ltd. Trifluoroacetic acid (TFA, 99%) was purchased from Alfa-Aesar and distilled under reduced pressure before use. 2,4,6-Trinitrobenzene sulfonic acid (TNBS) was supplied by Sigma-Aldrich. All Other reagents and solvents were of analytical grade and used as received without further purification.

Synthesis of α-CD-hydrazide-ALA segement

Synthesis of α-CD-hydrazide

α-CD-hydrazide was synthesized according to our previous research[1]. Briefly, α-CD (5 g, 5.1 mmol) dissolved in 50mL dry dimethylformamid (DMF) was activated with 4-Nitrophenyl chloroformate (6.23g, 30.9 mmol) in the presence of triethylamine (4.27 mL, 28.4 mmol) and

under N_2 atmosphere. The resulting mixture was allowed to stir at room temperature for 24 h and then filtered to remove the byproduct. The product was purified by precipitating in methanol for three times and dried in vacuum at 35 ºC overnight. To get α-CD-hydrazide, the activated α-CD (α-CD-NC, 2.5 g) was dissolved in 5 mL DMF and then reacted with hydrazine (7 mL) in nitrogen at 50 ºC for 24 h. The product was obtained by precipitation and dried in vacuum at 35 ºC overnight.

Synthesis of α-CD-hydrazide-ALA

α-CD-hydrazide(100 mg) and ALA·HCl(40 mg) was dissolved in 5mL DMSO. 5 μL TFA was added after stirring for 5 min. After reacting for another 48 h in the dark at room temperature, the solution was dialyszed against distilled water (MWCO 500) for 2 days and lyophilized to obtain crude α-CD-hydrazide-ALA.

Preparation of supramolecular prodrug micelles

α-CD-hydrazide-ALA (14.8 mg) was dissolved in 3 mL deionized water and PEG-R6H4 (2 mg) dissolved in 2 mL deionized was added dropwise. After vigorously stirred for 2 h, the mixture was subjected to sonication for 20 min and dialyzed against distilled water (MWCO 3500) for 2 days to remove byproduct and stored at 4 ºC prior to use. To determine ALA loading content of the micelles, 1 mL micellar solution was treated with 1 N HCl for 24 h and lyophilized to remove water. Then, 1 mL anhydrous methanol was added into the dry product, in which α-CD-hydrazide and PEG-R6H4 were insoluble. ALA methanol solution was obtained by centrifugation. After removing the solvent by reduced pressure distillation, the product was diluted with 3 mL distilled water. At last, the ALA content was tested using a 2,4,6-trinitrobenzene sulfonic acid (TNBS) kit TNBS.[2]

Control release of ALA from the supramolecular prodrug micelles under different pH

The release of ALA was conducted by a dialysis method. Briefly, 2 mL of micellar solution was transferred to a dialysis bag (MWCO 500 Da) and immersed in jar containing 8 mL PB buffer under continually stirring(100 rpm) at 37 ºC. The ALA release experiments were carried out at pH 7.4, 6.5 and 5.5, respectively. At predetermined time intervals, 1 mL liquid in the jars was taken out and 1 mL fresh buffer of each pH was added into the jars. The release experiments lasted for three days. The collected liquid was reacted with TNBS as an assay reagent to detect ALA. The assay was performed using a TNBS kit according to the manufacturer's instructions, and a serial of gradient ALA standard solutions was used to prepare the calibration curve.

Cell Culture

Human hepatocellular carcinoma cells (HepG2 cells) were cultured at 37 °C and 5% CO_2 environment. The cell culture medium used was Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin.

Fluorescent microscopy and flow cytometry tested cellular uptake, the release and conversion of ALA and the ROS generation of the micelles.

Fluorescent microscopy

HepG2 cells were inoculated into 24 well plates at 2 × 10^4 cells per well in DMEM medium. After 24 h incubation, the medium was replaced with fresh serum-free medium of different pH (7.4 and 6.5). After two more hours' incubation, the cells were treated with prodrug micelles (20 μg /mL ALA) for 2, 4 h respectively. Then, the medium was removed and the cells were washed by PBS for three times. To test the cellular uptake of micelles, the cells were fixed with 4 % paraformaldehyde. Finally, the images were obtained immediately by Fluorescent microscopy.

To study the ROS generation of the micelles, the cells were further incubated with 10 μM dichlorofluorescein diacetate (DCF-DA) in serum-free medium for 30 min, then, washed by PBS for three times followed by irradiating with 635 laser for 60 s, Finally, DCF-DA fluorescence was imaged immediately.

Flow cytometry

For flow cytometry, HepG2 cells were inoculated into 24 well plates at 2×10^5 cells per well, other processes were same as the fluorescent test. After washing by PBS, cells were treated with trypsin and centrifuged for 5 min at 1000 rpm. Then the cells were suspended in 0.5 mL of PBS and analyzed using a FACScan flow cytometer.

Photo-Cytotoxicity by MTT Assay

96-well microtiter plates seeded with 1×10^4 HepG2 cells were incubated overnight at 37 °C with 5% CO_2. Cells were subsequently exposed to ALA micelles at predetermined concentrations in fresh serum-free medium of different pH (7.4 and 6.5) for 4 hours. After washed by PBS, the cells were irradiated with 635 laser (0.3 W cm^{-2}, 2 min) and incubated overnight with serum-free medium. Then, 20 μL MTT (5 mg mL^{-1}) was added to each well and the cells were further cultured at 37 °C for 4 h. Finally, the media was aspirated and replaced with 150 μL of DMSO and formazan absorbance was determined by a microplate reader (MODEL 550, Bio Rad) at 490 nm. The cells without any treatment were used as control. To validate the proposed time-controlled PDT, cells treated with micelles (9 μg mL^{-1}) incubating in different pH culture medium were exposed to irradiation with 635 nm light for different durations. The Cell viability was tested as mentioned above.

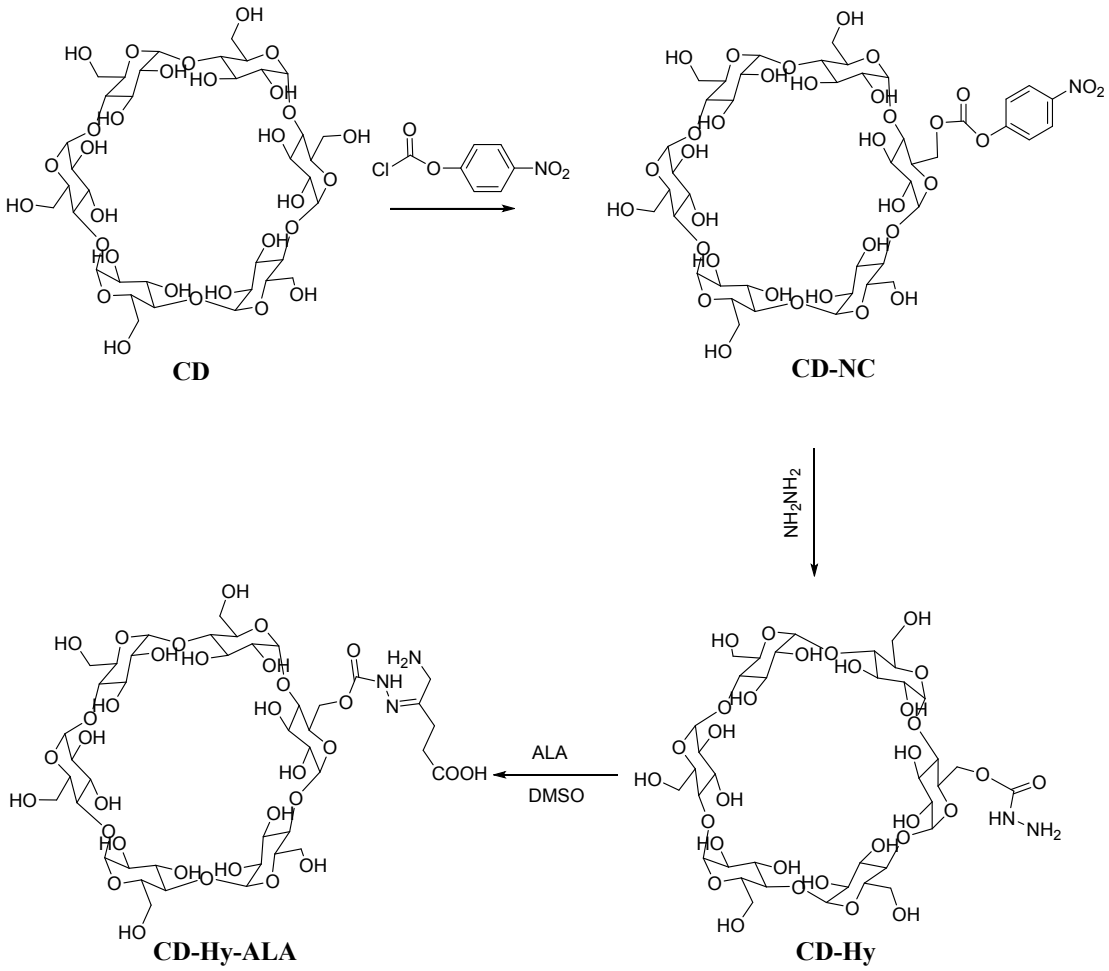
Characterizations

The ^{1}H NMR spectra were recorded on a Bruker DMX500 spectrometer operating at 500 MHz using DMSO-d_6 or D_2O as the solvent. The size and Zeta potential of the prodrug micelles in different buffer were measured by Zetasizer Nano-ZS from Malvern Instruments equipped with a He-Ne laser at wavelength of 633 nm with an angle of 173^{o} (25 °C). The sizes and morphologies of the resultant samples were also characterized by JEM-1230 transmission electron microscopy (TEM) at an accelerating voltage of 80 kV, whereby a small drop of sample solution was deposited onto a carbon-coated copper TEM grid (230 mesh) and dried at room temperature at atmospheric pressure. The release of ALA was test by a Shimadzu UV-2505 spectrophotometer. Fluorescence images were acquired by using a Perkin-Elmer LS 55 fluorescence spectrometer. Flow cytometry was analyzed by FACSCalibur flow cytometer.

References

1 Y. Wang, H. B, Wang, Y. J. Chen, X. S. Lv, Q. Jin, J. Ji, *Chem Commun*, 2013, **49**, 7123.

2 S. J. Yang, C. F. Lin, M. L. Kuo, C. T. Tan, *Biomacromolecules*, 2013, **14**, 3183.



Scheme. S1 Detailed synthetic route of α-CD-hydrazide-ALA

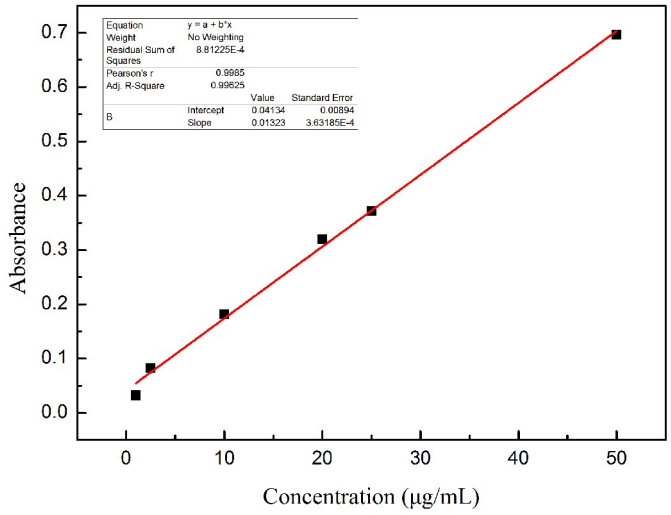


Fig. S1 The calibration curve of ALA by using TNBS kit.

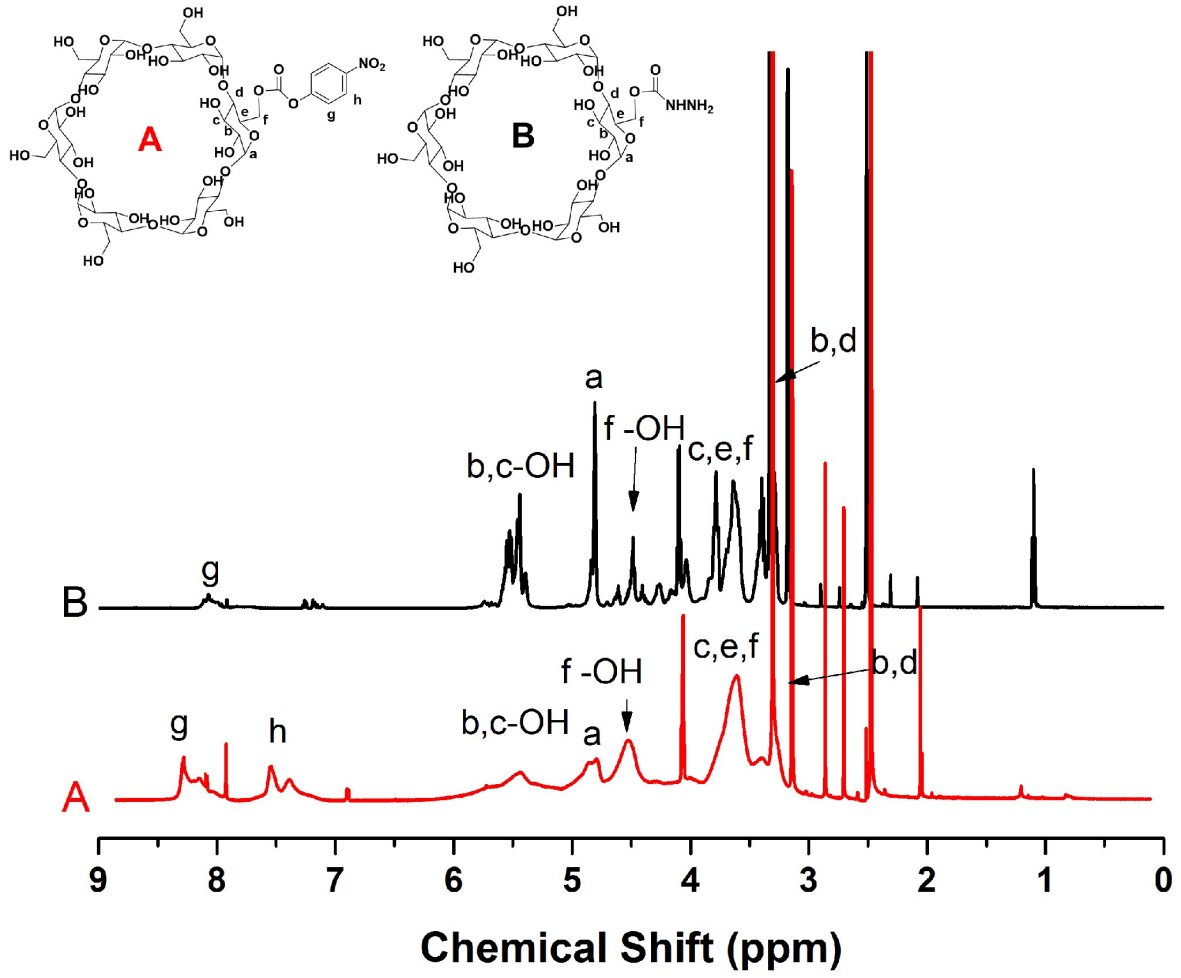


Fig. S2 ^{1}H NMR spectra of α-CD-NC (A), α-CD-hydrazide (B) (500 MHz, DMSO-d_6)

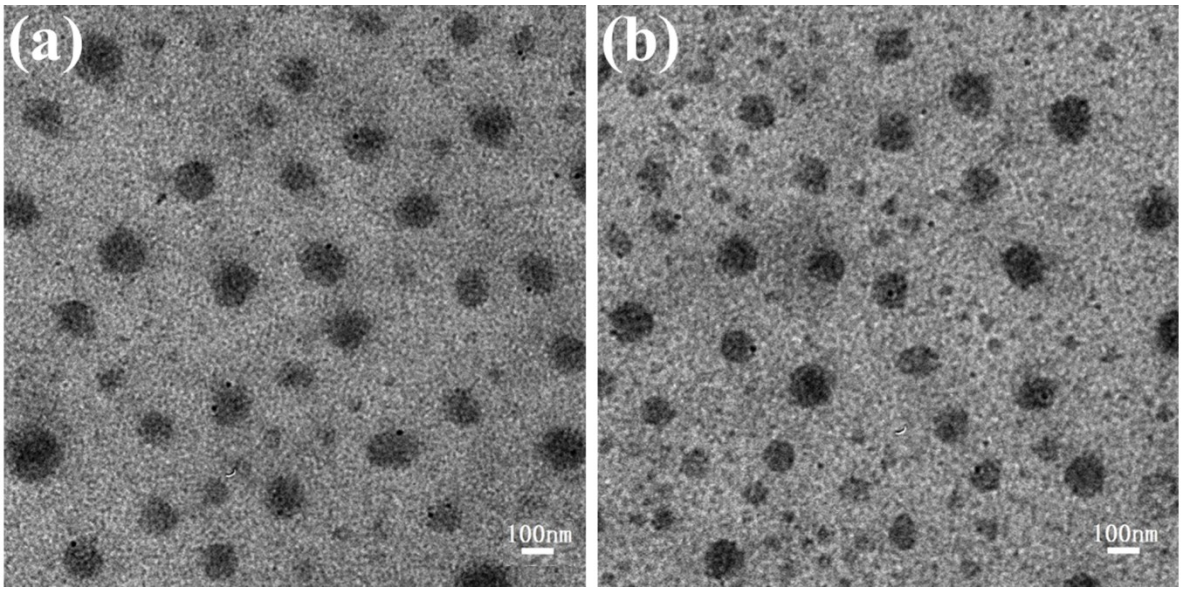


Fig. S3 TEM images of Prodrug micelles at (a) pH 6.5 and (b) pH 7.4

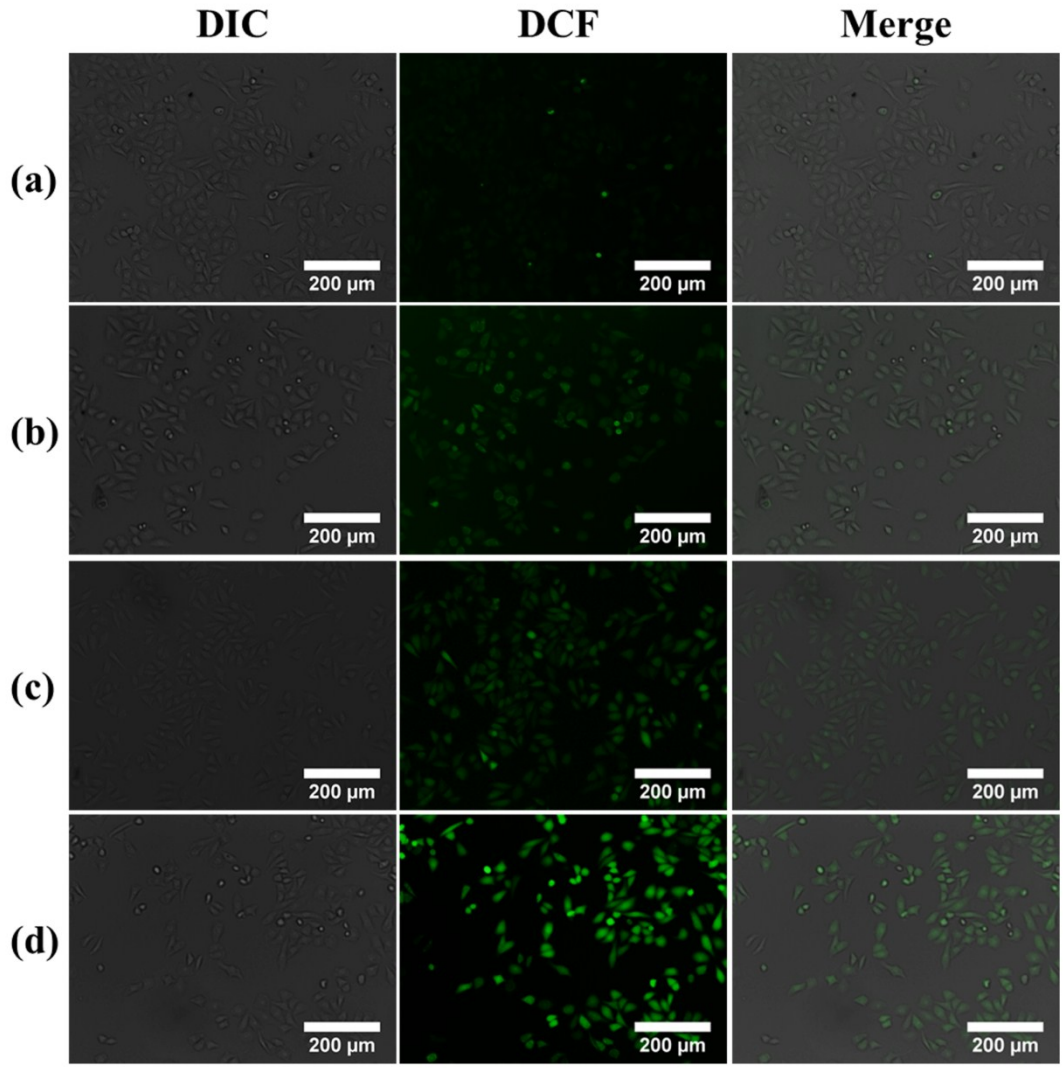


Fig. S4 Singlet oxygen production detected by fluorescence of DCF-DA in HepG2 cells exposed to prodrug (10 μg mL^{-1} ALA) and irradiated with 635 nm light (0.1 W cm^{-2}, 1 min). Images from left to right are DIC channel, DCF channel and a merge of the two channels: (a) pH 7.4, 2 h;(b) pH 6.5, 4 h; (c) pH 6.5, 2 h ;(d) pH 6.5, 4 h.

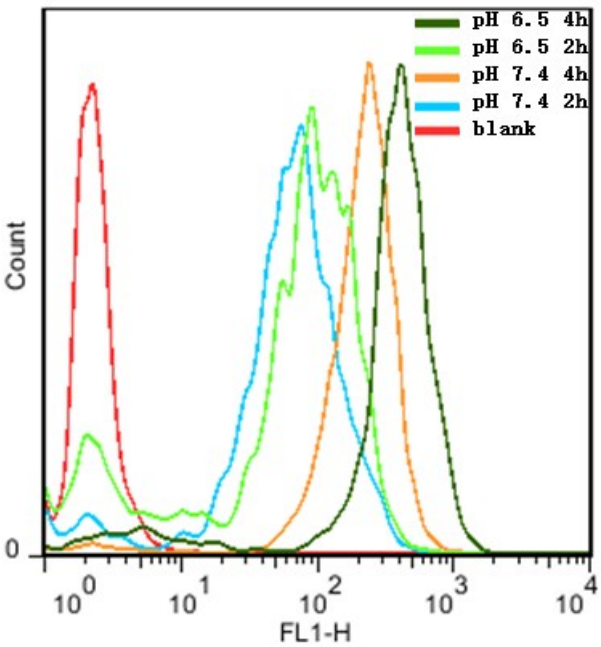


Fig. S5 Flow cytometric profiles of HepG2 cells incubated with prodrug micelles(10 μg mL^{-1}) for 2 h or 4 h in different pH culture medium after stained with DCF-DA and irradiation (635 nm ,0.1 W cm^{-2}, 1 min)