Enzyme-triggered Dis-shielding of Nanoparticles and Positive-charged Mediated Lysosome Escaping for Chemo/Photo-Combination Therapy

X.Q. Zhang,[a,b] S. S. Cai ,[b] Y.M. He,[b] M. Zhang,[a,b] J. Cao,[b,] H. Mei,[b] S. Li *[a] and B. He [b]*

[a] College of Chemical Engineering, Sichuan University, Chengdu 610065 China

[b] National Engineering Research Center for Biomaterials, Sichuan University, 29# Wangjiang Road, Chengdu 610064 China

Materials and Methods

Materials. Poly(ethylene glycol) methyl ether (mPEG, Mw=5,000 g·mol^{-1}) (mPEG5k) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich Co. (Steinheim, Germany); Triphosgene, 4-Toluene sulfochloride, protoporphyrin IX, and N-Hydroxysuccinimide (NHS) were obtained from Adamas Reagent Co., Ltd; N_6-Cbz-L-Lysine and Hydrogen bromide (33 wt.% in Acetic acid) were purchased from Energy Chemical (China) and doxorubicin hydrochloride (DOX·HCl) was purchased from Shanghai Yingxuan Chempharm Co., Ltd.(China). N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and HAase were purchased from HEOWNS. and Sigma. Hyaluronic acid (HA, Mw = 40,000) was purchased from BLOOMAGE FREDA BIOPHARM Co., Ltd. The reagents mentioned above were used as received. Chloroform-d_3 ($CDCl_3$) and dimethyl sulfoxide-d_6 were purchased from Sigma-Aldrich Co. Dialysis membranes (Spectra/Por 3 dialysis sack, 3.5K, 2.0 K MWCO) were commercially available from Spectrum/Por (Houston, TX, USA). Cell lines, A549 and 4T1 were purchased from the Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum and penicillin-streptomycin were used for cell tests, which were purchased from HyClone Inc. Lysosome tracker (green) was purchased from GeneCopoeia, Inc. All of those reagents were used as received.

BALB/c mice (Femal, 20 ± 2g) were purchased from Dashuo Biotechnology Co., Ltd, (Chengdu, China) and animal experiments were carried out under the approval of the experiments animal administrative committee of Sichuan University.

Synthesis of mPEG-PLLys-PpIX block copolymers. The amination of terminal hydroxyl groups of mPEG (mPEG-OH) and the synthesis of the N-carboxyanhydrides (NCA) of N_6-Cbz-L-Lysine were performed according to our previous study.[1, 2] In brief, a certain amount of N_6-Cbz-L-Lysine (10.00 g) was added to the anhydrous THF (100 mL) under nitrogen atmosphere, and then a solution of triphosgene (13.76 g) in THF (50 mL) was added dropwise to the mixture in an oil bath at 50℃. The termination of the reaction was carried out when the solution became clear. After the solution was condensed using rotary evaporator, it was precipitated in large amounts of anhydrous hexane three times to remove the residual triphosgene. The white powder (NCA cyclic monomer) was vacuum-dried at room temperature for 24 h.

mPEG5k-NH_2 (2.00 g) and NCA (4.90 g) were mixed in CH_2Cl_2 (100 mL) and stirred at room temperature under nitrogen atmosphere. After 3 days, the mixture was poured into a large amounts of anhydrous diethyl ether, then the powder was collected through filtration and vacuum-dried at room temperature. The obtained powder was dissolved in trifluoroacetic acid until clear, and then hydrogen bromide was added dropwise. After stirring for 1 h at room temperature, the mixture was washed by a large of anhydrous diethyl ether for three times. Then the final product (mPEG-PLys) was obtained by freeze-drying after dialysis (MWCO=3,500 Da) with pure water for 4 days.

PpIX (0.67 g), EDC (2.384 g), NHS (0.143 g) were dissolved in anhydrous DMSO (15 mL) and stirred for 3 h at room temperature to activate carboxyl group of PpIX, and then the solution was added dropwise to the solution of mPEG-PLys (1.00 g). After stirring 48 h, the solution was concentrated and further purified by dialysis (MWCO = 3,500 Da). The final product (mPEG-PLLys-PpIX) was obtained by freeze-drying. The structures of those polymers were characterized by the ^{1}HNMR spectra using $CDCl_3$ and DMSO-d_6 as solvents,

and 0.5% tetramethylsilane was used as the internal standard.

Synthesis of HA-DOX. HA (0.25 g), EDC (0.415 g), and NHS (0.025 g) were dissolved in the mixture solutions of DMSO/H_2O (1:1, v:v) and stirred for 3 h at room temperature. After that, DOX (0.107 g) was added dropwise for stirring another 48 h, the solution was concentrated and the crude product was further purified by dialysis (MWCO = 3,500 Da). HA-DOX was obtained by freeze-drying. The structure of the polymer was characterized by ^{1}HNMR spectra.

Preparation of Nanoparticles (NPs). 10 mg of mPEG-PLLys-PpIX block polymer dissolved in 0.5 mL of DMSO was added dropwise into 10 mL deionized water. After stirring overnight, the solution was transferred into dialysis tubing (MWCO = 2,000) and dialyzed against deionized water for 8 h to thoroughly remove the organic solvent. The mPEG-PLys-PpIX micellar solution was obtained by centrifugation and then HA-DOX dissolved in distilled water (6 mg/mL) was added and mixed 60 s with a vortex to get HA-DOX/mPEG-PLys-PpIX (HD@EGPpIX) micellar solution. The size and zeta potential of nanoparticles were determined by a dynamic light scattering (DLS) spectrometer (Malvern Zetasizer Nano ZS) at the temperature of 25℃. The morphologies of NPs were characterized by transmission electron microscope (TEM)

Enzyme-triggered Dis-shielding and Charge Reverse of NPs. The size and morphology of HD@EGPpIX micellar solution in response to different concentrations of HAase was monitored by DLS and TEM measurements. First, 1 mL of HD@EGPpIX was incubated with different concentrations of HAase at 37℃. A micellar solution without HAase was used as the control. At predetermined intervals, the sizes and zeta potential of the polymeric NPs were determined by DLS and TEM.

In Vitro Drug Release. HD@EGPpIX micellar solution ([DOX] = 50 μg/mL) was placed in dialysis tubing (MWCO = 2,000). The tubings were then immersed in brown vials containing 20 mL of different media (PBS, ionic strength = 0.01M), pH 5.5 (PBS, ionic strength = 0.01M), pH 5.5 (PBS, ionic strength = 0.01M) with HAase (100 IU/mL-250 IU/mL) and the vials were placed in a shaking bed at the temperature of 37 ℃. At the prescribed intervals, a fixed volume (1 mL) of sample media was taken out, and the same volume of released media was added to the vails. The released DOX was determined by a fluorescence detector with excitation and emission wavelengths of 483 nm and 550 nm, respectively. The release experiments were conducted in triplicate, and the results were demonstrated as mean ± SD.

In Vitro Evaluation of Antitumor Effects. Briefly, 4T1 cells (4×10^4 cells/mL) and A549 cells (4×10^4 cells/mL) were separately seeded in 96-well plates. After 12 h incubation at 37℃ in 5% CO_2 atmosphere, the culture medium was removed and replaced with 100 μL of DOX·HCl and NPs with different concentrations of DOX and incubated for another 48 h. MTT (5 mg/mL) diluted 10 times with culture medium was added into each well, and then the plates were incubated at 37℃ for 4 h. After the medium was replaced by 100 μL of dimethyl sulfoxide, the absorbance was measured by Thermo Scientific MK3 microplate reader (Thermo fisher Co., USA) at 490 nm.

Cellular Uptake. 4T1 cells with a cell density of 2×10^4 cells/mL was seeded on glass dishes (d = 35 mm). After being cultured for 24 h, 1 mL of DOX·HCl or HD@EGPpIX ([DOX] = 10 μg/mL) was added to the cells with/without pre-incubation of HA. After another 2 h and 4 h incubation, the cells were stained with LysoBeacon™ Green (LysoBeacon™ Green) for 40 min at defined time intervals. Subsequently, the medium was removed and the cells were washed with cold PBS three times, then 1mL of PBS was added to each dish, and fluorescent distribution was observed using CLSM (Leica TCP SP5). For a quantitative assay, 4T1 cells were seeded onto a cover glass in a 6-well plate (1×10^5 cells/mL). After 24 h incubation, different NPs were put into the wells for 2 h and 4 h respectively at a concentration of 10 g/mL (DOX) per well. Then, the cells were

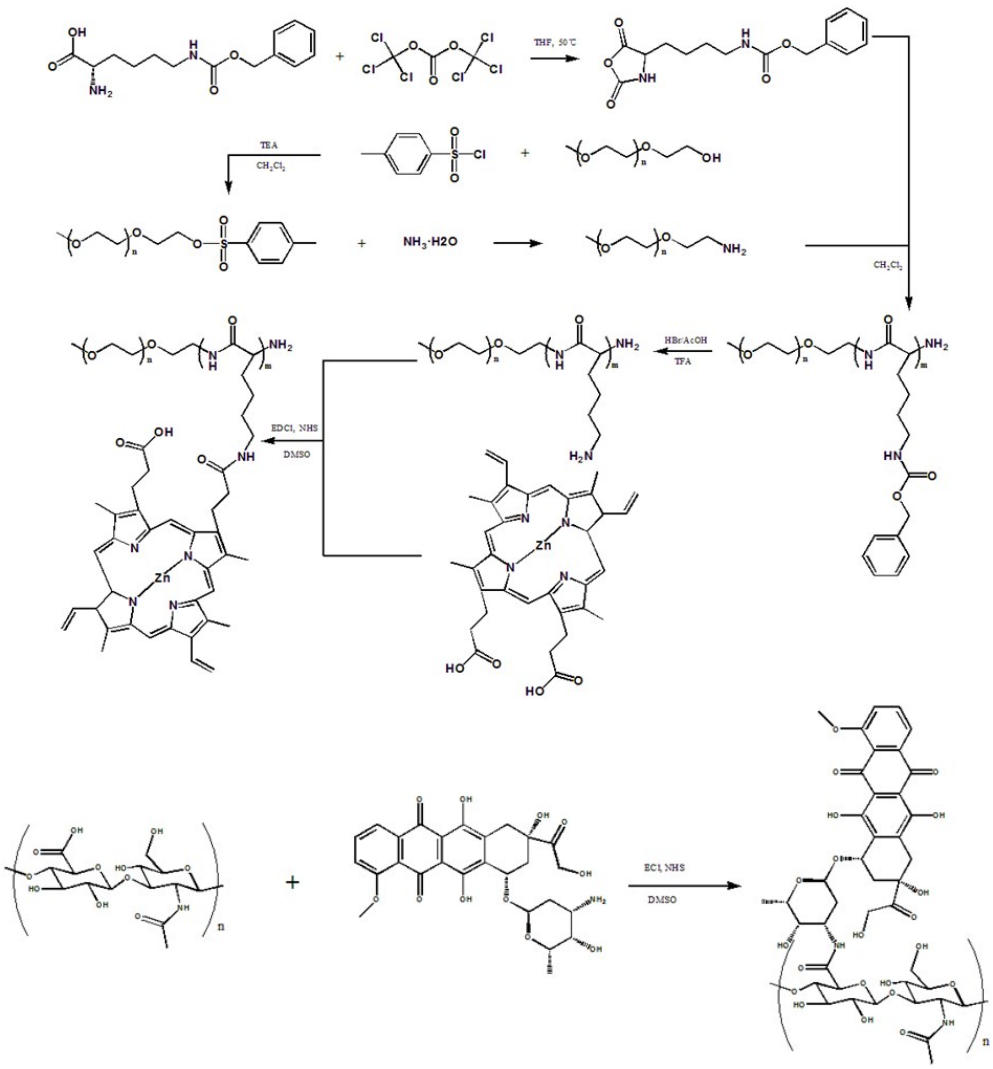
processed and observed by flow cytometry (BD FACSCalibur, USA)

Lysosomal distribution. 4T1 cells with a cell density of 2×10^4 cells/mL was seeded on glass dishes (d = 35 mm) and incubated for 24 h, then HD@EGPpIX ([DOX] = 10 μg/mL, with pretreatment of HAase) was added to the cells. After another 2 h, 4 h, and 6 h incubation, lysosomes were stained with LysoBeacon™ Green. Subsequently, the cells were washed with cold PBS three times, then 1mL of PBS was added to each dish used for CLSM observation (Leica TCP SP5).

In vitro evaluation of ROS generation. To evaluate the ROS generation of HD@EGPpIX, Reactive Oxygen Species Assay Kit (DCFH-DA, Beyotime Biotechnology Co., Ltd., Shanghai) was used to detect the level of ROS generation in 4T1 cells. 4T1 cells (2×10^4 cells/mL) were seeded on glass dishes (d = 35 mm) and incubated for 24 h, then different DOX concentrations of NPs (5 μg/mL, 2 μg/mL and 1 μg/mL) were added to the cells. After incubation for 4 h, the medium was replaced by fresh culture medium (1 mL) containing DCFH-DA. The dishes were exposed to the laser of 660 nm for 2 min after further incubating for 30 min. Then the cells were washed three times with cold PBS and observed using CLSM to detect the fluorescence of DCF (Ex = 488 nm, Em = 525 nm). Meanwhile, for quantitative determination of ROS generation, 4T1 cells (1×10^5 cells/mL) were seeded in 6-wells plates and cultured for 24 h. Then cells were cultured with NPs as described above. After cells were trypsinized and washed with cold PBS twice, the cells were centrifuged and resuspended in 0.4 mL of PBS. The fluorescence intensities of DCF and DOX (Ex = 483 nm, Em = 550 nm) were analyzed by a flow cytometer (BD FACSCalibur, USA).

In Vivo Imaging.

The animal models were established by injecting 4T1 cells (5×10^6 cells suspended in 100 μL cells culture medium) subcutaneously into the left flank of the BALB/c mice. After the tumor volume reached 150-200 mm^3, DiR/HD@EGPpIX and DiR/EGPpIX NPs (0.24 mg DiR/kg) were injected into the 4T1-bearing mice by the tail vein. After injected for 12, 24, and 36 h, the mice were imaged using the Lumina III Imaging System (PerkinElmer, USA).



Scheme S1. The synthetic routes of mPEG-PLLys-PpIX copolymer and HA-DOX.

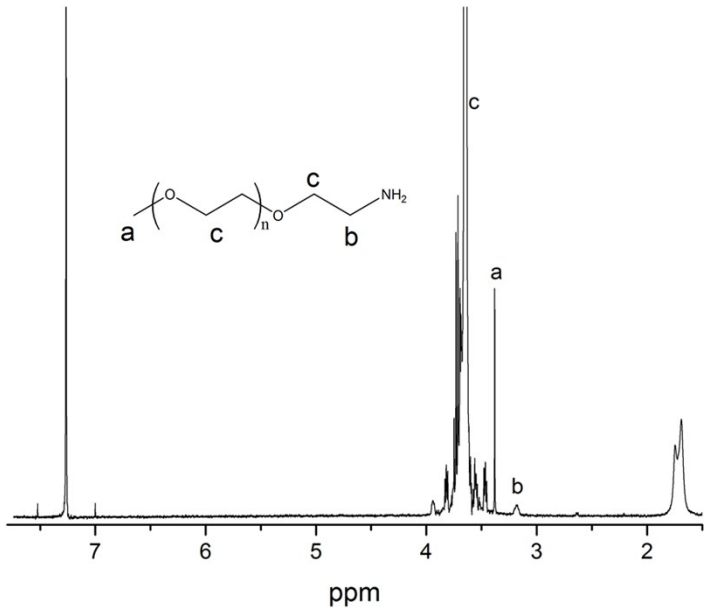


Fig S1. The ^{1}HNMR spectrum of mPEG-NH_2.

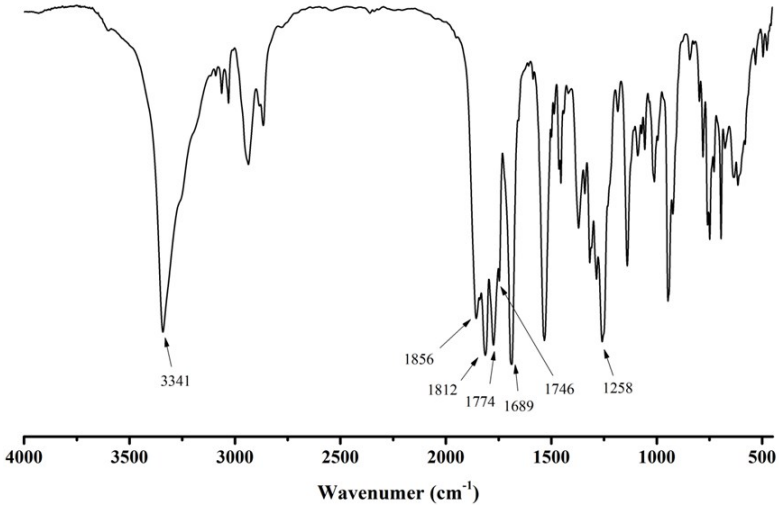


Fig S2. The FTIR spectrum of mPEG-NH_2.

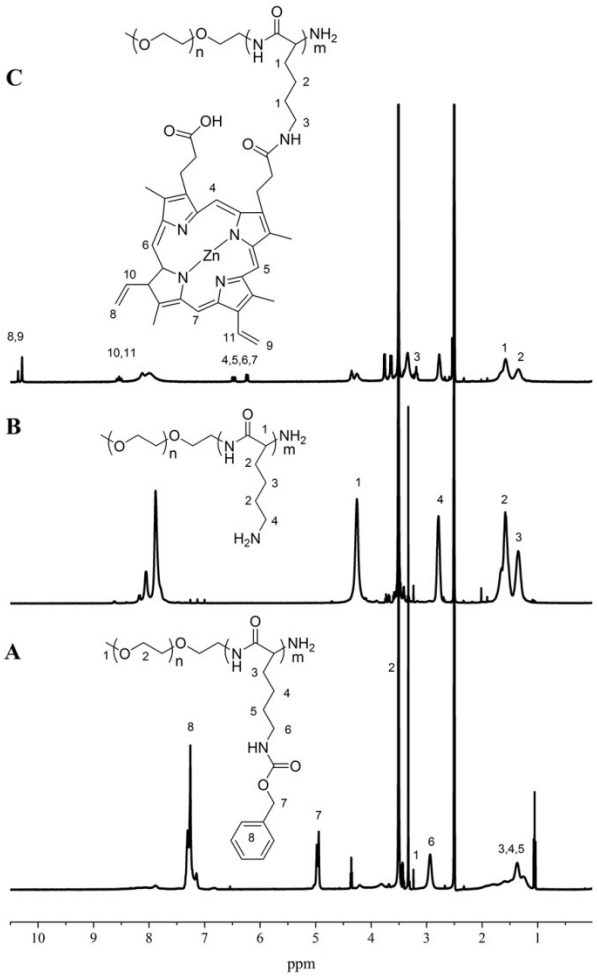


Fig S3. [1]HNMR spectra of mPEG-PLLBz, mPEG-PLLys and mPEG-PLLys-PpIX copolymers.

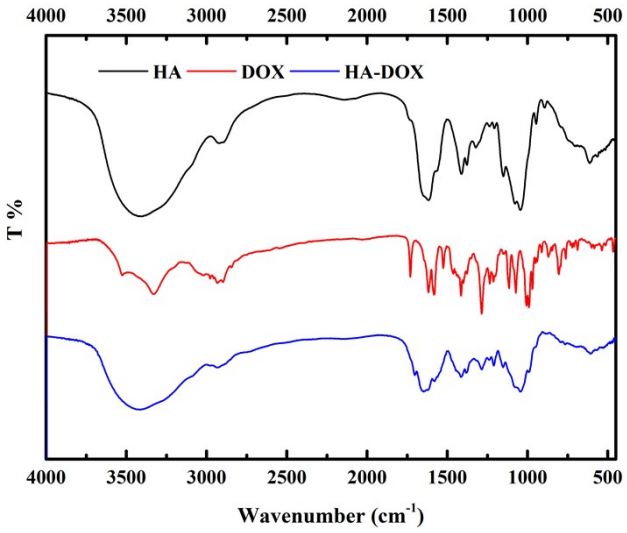


Fig S4. FTIR spectra of HA, DOX and HA-DOX.

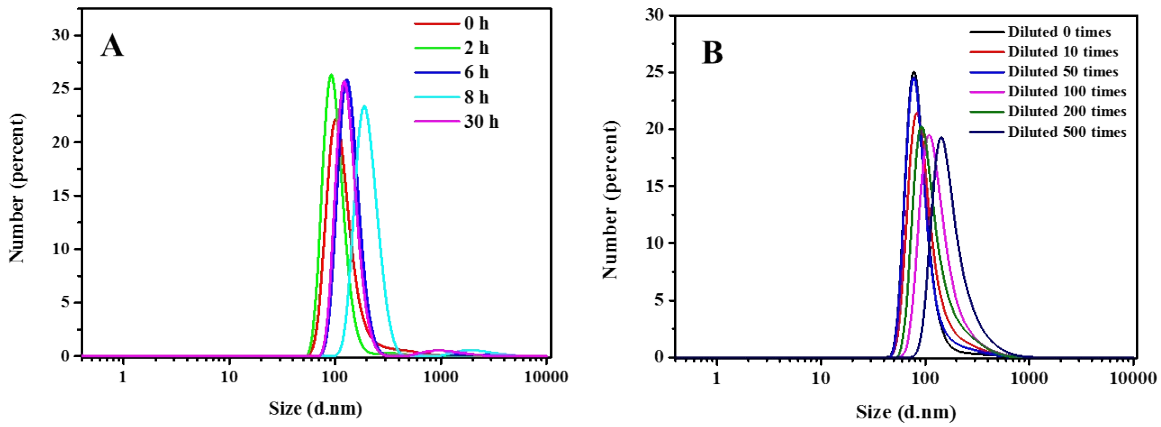


Fig S5. The stability of HD@EPpIX NPs incubated with culture medium (A) and diluted with water (B).

Table S1. Release exponent (n), rate constant (k), and correlation coefficient (R2) HD@EGPpIX NPs

	pH 7.4	pH 5.5	pH 5.5 + HAase
n	0.225	0.213	0.227
k	0.189	0.246	0.272
R^2	0.980	0.975	0.978

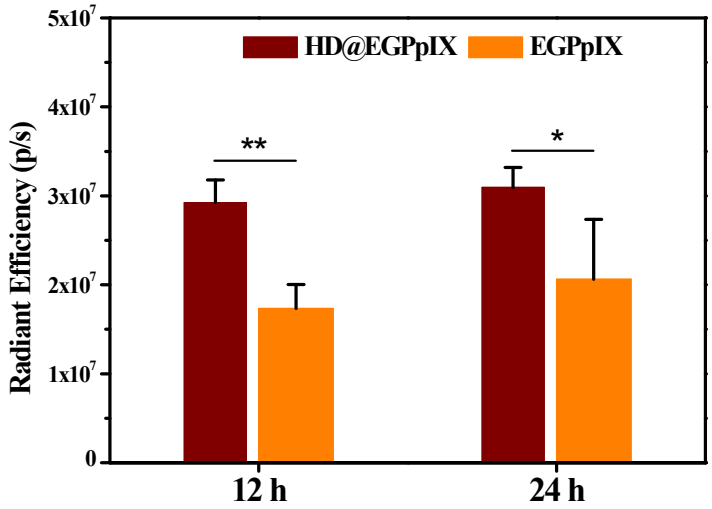


Fig S6. ROI analysis of DiR fluorescence signals from the tumors (means ± SD, n = 3, 0.01 < *p < 0.05, 0.001 < **p < 0.01).

1. J. Cao, T. Su, L. G. Zhang, R. Liu, G. Wang, B. He and Z. W. Gu, *Int J Pharmaceut*, 2014, **471**, 28-36.
2. T. T. Xu, J. H. Li, F. R. Cheng, Y. X. Zhang, J. Cao, W. X. Gao and B. He, *Chinese Chem Lett*, 2017, **28**, 1885-1888.