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Electronic Supplementary Information

Therapeutic Iminoboronate-Based Polymersomes with Cu(II)-Mediated Fenton Reaction-Enhanced ROS-Response

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Abstract: Reactive oxygen species (ROS)-responsive prodrug nanoplatform (ROS-RPN) can controllably deliver drugs triggered by the overproduced ROS in tumor tissues. However, ROS-RPN may not work due to insufficient concentrations of ROS, and thus it is imperative to improve the sensitivity of ROS-RPN. Herein, we demonstrate a new design of high sensitivity ROS-RPN. Knowing that there are 2-3 times of Cu(II) in tumors than in normal tissues, and that the Cu(II)-mediated Fenton-like reaction can catalyze H_2O_2 to generate highly reactive •OH under neutral pH condition, therapeutic polymersomes of a metallisable triamine-centered iminoboronate-functionalized amphiphilic starlike prodrug (N_3-(OEG-IBCAPE)$_4$) are prepared to show the Cu(II)-mediated Fenton reaction-enhanced ROS response. Indeed, the chelating of Cu(II) ions to the triamines in the prodrug results in the Fenton-like reaction and effectively produces •OH surrounding the nanoparticles, which leads to an enhanced •OH-triggered oxidation of iminoboronate moieties with an improved release of parent CAPE molecules. The *in vitro* investigation further confirms the high therapeutic efficacy of the iminoboronate-terminalized starlike prodrug.

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1. Materials and Methods

Poly(ethylene glycol) diglycidyl ether (PEGDGE, Mn = 394), 2, 2'-Diamino-N-methyldiethylamine (DNMDA), cysteamine, formylphenylboronic acid (FPBA), caffeic acid phenethyl ester (CAPE), tributylphosphine, cupric acetate monohydrate ($Cu(CH_3COO)_2 \cdot H_2O$), H_2O_2 (30%), dimethylsulfoxide (DMSO) were purchased from Aladdin and used without further purification. CCK-8 was obtained from Sigma Chemical Co. (USA). Hochest-33342 (H1399), LysoTracker™ Green DND-26 (L7526) and CellROX™ Deep Red Reagent (C10422) were obtained from Thermo Fisher Scientific (USA). Ultrapure water was prepared utilizing a FDY-1002-UV-P purification system.

^{1}H-NMR (400 MHz) spectra were investigated by a Bruker spectrometer with $CDCl_3$ as solvent. Absorption spectra were acquired on Evolution 220 spectrophotometer at 25 ^{o}C with a thermostatted cuvette holder. Fluorescence emission spectra were measured with a PTI fluorescence master system at 25 ^{o}C with a thermostatted cuvette holder. The slit width was maintained at 3 nm for excitation and 1 nm for emission, respectively. Dynamic light scattering (DLS) measurements were performed with a Malvern Nano-ZS90 instrument to determine the average diameter and size distribution of the micelles at 25 ^{o}C. Transmission electron microscopy (TEM) observations were carried out using a JEM-2100 microscope. The samples were prepared by dropping the micellar solution onto copper specimen grids (400 mesh, supported with formvar/carbon film), and dried at room temperature overnight. Field-emission scanning electron microscope (FE-SEM) observations were conducted on a high resolution HITACHI SU8220 field-emission scanning electron microscopy. The samples for SEM observations were prepared without gold coating. AFM was taken with a CSPM5500A scanning probe microscope system (Beijing Nano-Instruments Co., Ltd.) in the tapping mode. Samples were prepared by dropping the micellar solution onto mica sheets and drying the sheets at room temperature overnight. Confocal laser scanning microscopy (CLSM) images were acquired using a Leica TCS SP5 microscope.

2. Polymer Synthesis

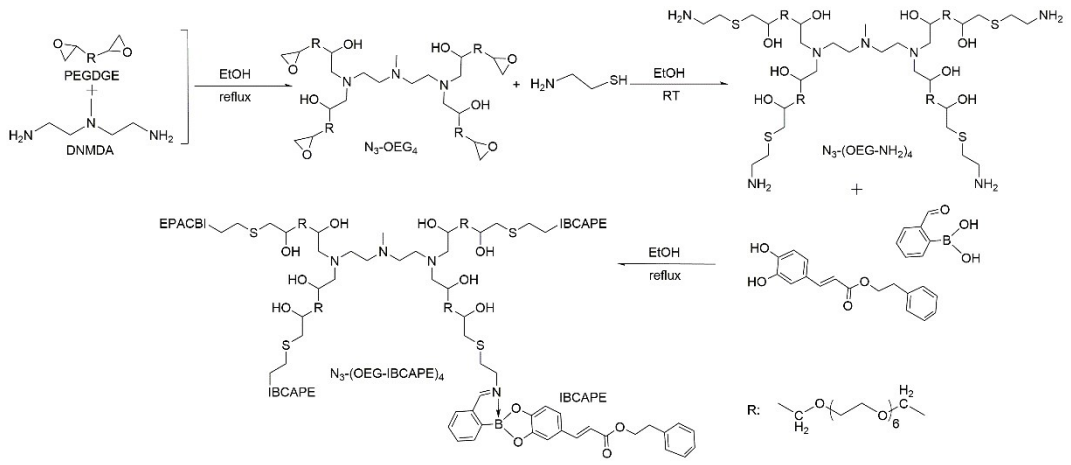


Figure S1. The synthetic route to the amphiphilic starlike prodrug of N_3-(OEG-IBCAPE)$_4$

N_3-(OEG-NH_2)$_4$ and N_3-(OEG-IBCAPE)$_4$ were synthesized according to our previous report work.[1] Figure S1 showed the synthetic route to the amphiphilic starlike prodrug of N_3-(OEG-IBCAPE)$_4$.

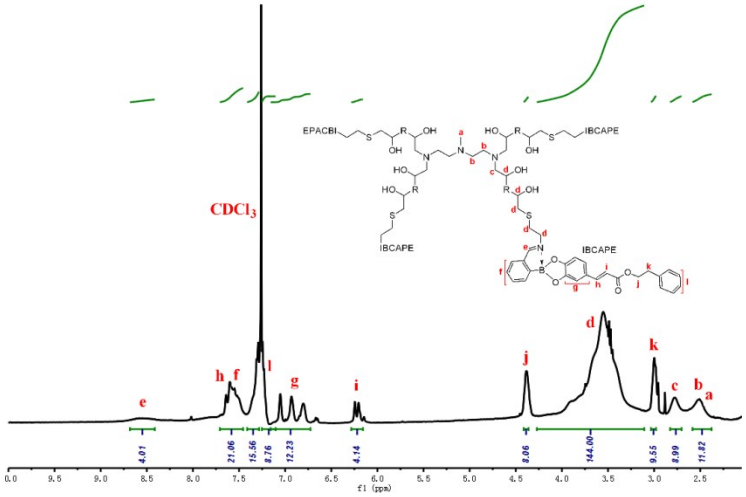


Figure S2. The ^{1}H-NMR spectrum of N_3-(OEG-IBCAPE)$_4$ in $CDCl_3$

^{1}H-NMR (400 MHz, $CDCl_3$, δ): 8.74-8.38 (-C=N-), 7.72-6.60 (Ar-H, Ar-CH_2=C-), 6.34-6.04 (Ar-C=CH-), 4.42-4.35 (-C(O)OCH_2-), 4.29-3.07 (-OCH_2CH_2O-, -CH(OH)$CH_2SCH_2CH_2NH_2$), 3.04-2.98 (-C(O)OC-CH_2-Ar), 2.82-2.71 (-(HO)CHCH_2N<), 2.58-2.38(>NCH_2CH_2N<, >NCH_3). Anal. Calcd for $C_{181}H_{247}B_4N_7O_{52}S_4$: C, 61.68; H, 7.06; N, 2.78. Found: C, 61.65; H, 7.11; N, 2.70.

3. Polymersomes Preparation

In the polymersomes preparation experiment, N_3-(OEG-IBCAPE)$_4$ (9.0 mg), H_2O (6.0 mL) were added together and stirred for 12 h. Finally, the polymersomes solution was diluted to 1.5 mg mL^{-1} (with respect to polymer in water).

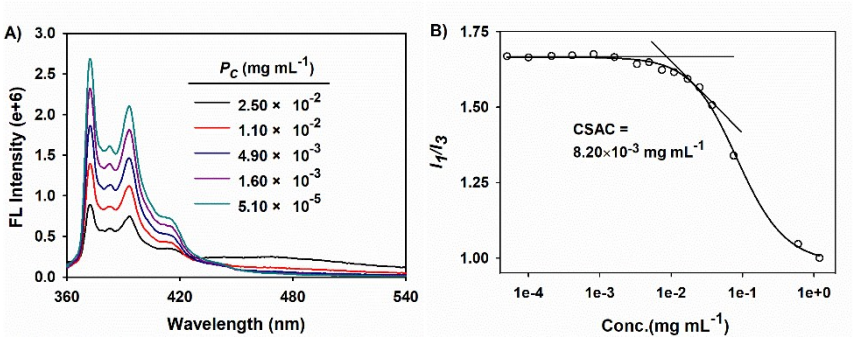


Figure S3. (A) Fluorescence emission spectra excitated at 340 nm as a function of polymer concentration in previously pyrene saturated aqueous solution. (B) Changes in the ratio of I_1/I_3 of pyrene emission as a function of polymer concentration.

In order to determine the critical self-assembly concentration (CSAC) of N_3-(OEG-IBCAPE)$_4$ in aqueous solution, the fluorescence pyrene probe technique was applied. As shown in Figure S3A, the fluorescence

emission spectra of N_3-(OEG-IBCAPE)$_4$ in pyrene saturated aqueous solution exhibited a characterized vibrational five peaks emission of pyrene. The ratio of the first (I_1 at 372 nm) and third peaks (I_3 at 382 nm) of pyrene emission remained constant up to a certain polymer concentration and decreases exponentially above it (see Figure S3B). This change reflected the onset of vesicle formation and the partitioning of the pyrene between the aqueous and vesicle phases. Therefore, the CSAC value for N_3-(OEG-IBCAPE)$_4$ in aqueous solution is 8.20 × 10^{-3} mg mL^{-1}.

4. Absorption and DLS Analysis of N_3-(OEG-IBCAPE)$_4$ Aqueous Solutions

During the experiment, the vesicular dispersion of N_3-(OEG-IBCAPE)$_4$ was sealed in a quartz cuvette at 25 °C with mild stirring, and different amounts of H_2O_2 were added into the solution. The checked dispersions were all kept at 0.3 mg mL^{-1} × 1.5 mL and pH 7.4.

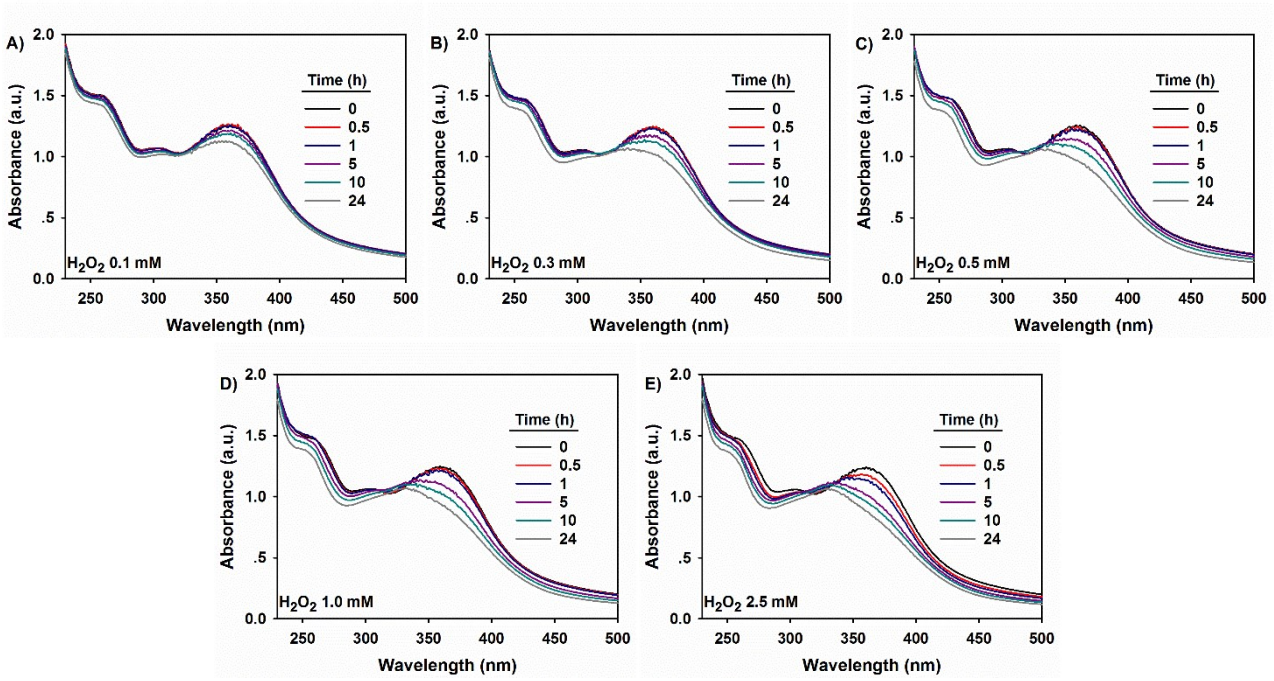


Figure S4. Absorption spectra of vesicular dispersion of N_3-(OEG-IBCAPE)$_4$ (0.3 mg mL^{-1} × 1.5 mL, pH 7.4) with H_2O_2 (A 0.1 mM, B 0.3 mM, C 0.5 mM, D 1.0 mM and E 2.5 mM) at different times.

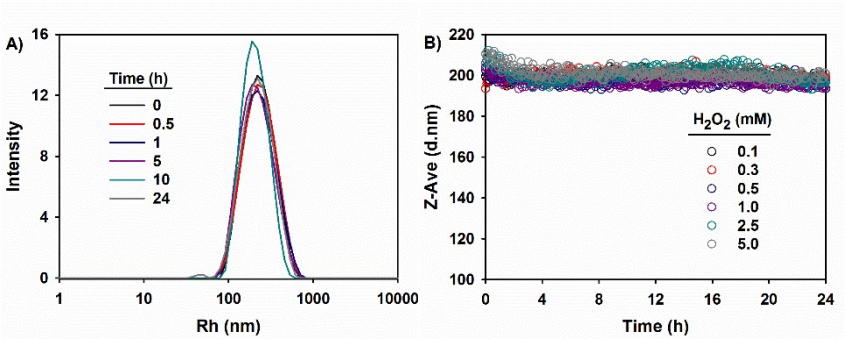


Figure S5. (A) The size distribution of N_3-(OEG-IBCAPE)$_4$ (0.3 mg mL^{-1} × 1.5 mL, pH 7.4) with H_2O_2 (5.0 mM) at different times. (B) Zeta average size changes of N_3-(OEG-IBCAPE)$_4$ (0.3 mg mL^{-1} × 1.5 mL, pH 7.4) with different concentrations of H_2O_2.

5. Absorption and DLS Analysis of N_3-(OEG-IBCAPE)$_4$-Cu Aqueous Solutions

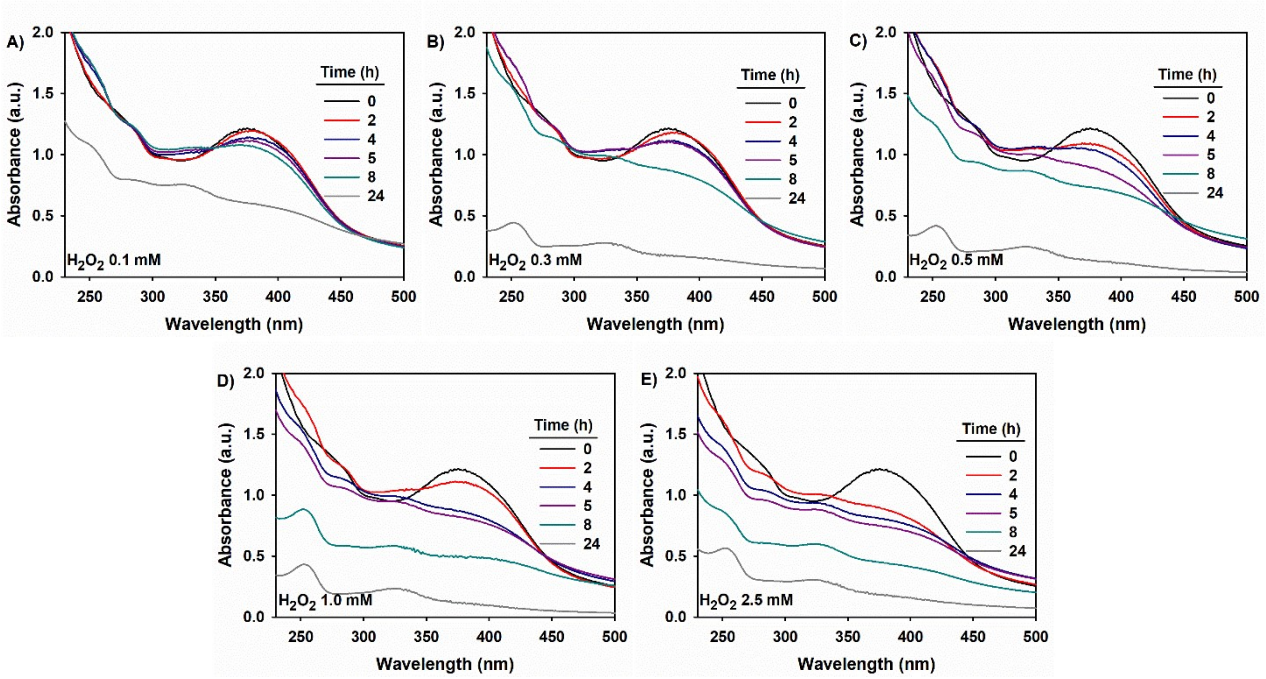


Figure S6. Absorption spectra of N_3-(OEG-IBCAPE)$_4$-Cu aqueous solution (0.3 mg mL^{-1} × 1.5 mL, Cu(II) (1.28 μg/mL)) with H_2O_2 (A 0.1 mM, B 0.3 mM, C 0.5 mM, D 1.0 mM and E 2.5 mM) at different times.

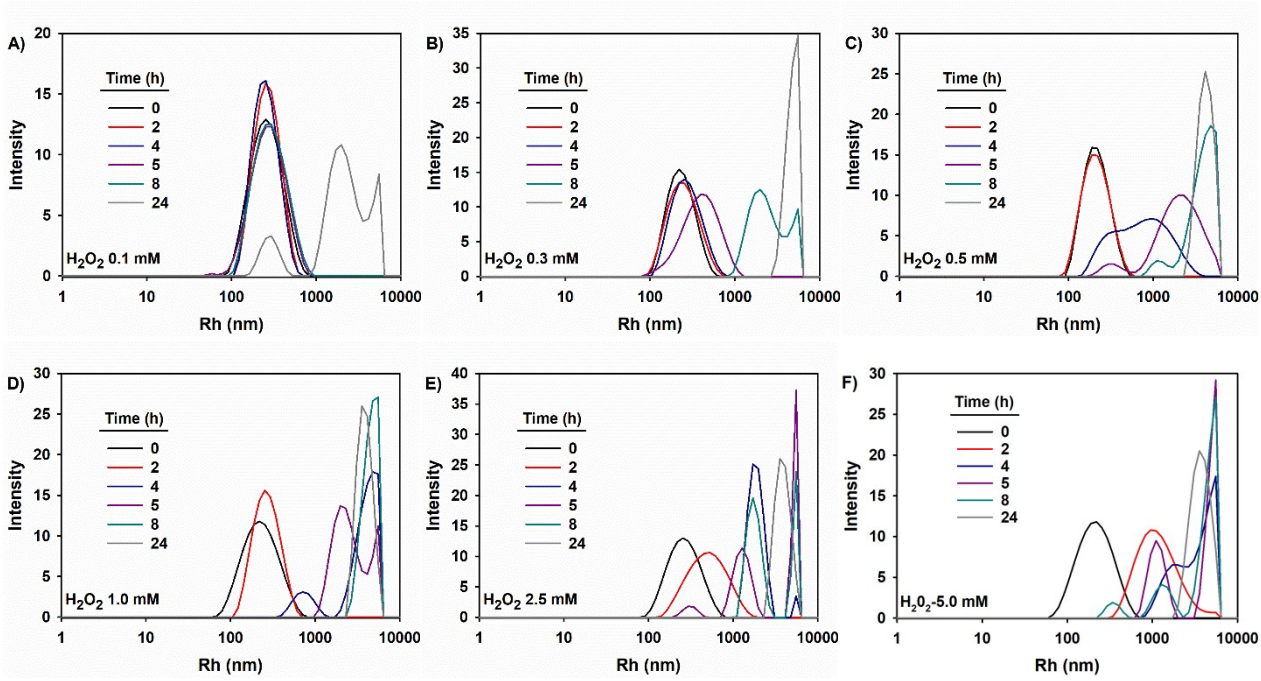


Figure S7. The size distribution of N_3-(OEG-IBCAPE)$_4$-Cu (0.3 mg mL^{-1} × 1.5 mL, Cu(II) (1.28 μg mL^{-1})) with H_2O_2 (A 0.1 mM, B 0.3 mM, C 0.5 mM, D 1.0 Mm, E 2.5 mM and F 5.0 mM) at different times.

During the experiment, the vesicular dispersion of N_3-(OEG-IBCAPE)$_4$-Cu aqueous solution (0.3 mg mL^{-1} × 1.5 mL, Cu(II) (1.28 μg mL^{-1}), pH 7.4)) was sealed in a quartz cuvette at 25 ^{o}C with mild stirring, and different amounts of H_2O_2 were added into the solution.

6. The Cellular Uptake of Polymersomes Observed with Confocal Laser Scanning Microscopy

N_3-(OEG-IBCAPE)$_4$ or N_3-(OEG-IBCAPE)$_4$-Cu was dispersed in the medium by slightly ultrasonication right before their introduction to the HepG2 cells. For confocal microscopy study, HepG2 cells were seeded at initial densities of 5 × 10^4 cells mL^{-1} in dishes (35 mm confocal imaging dish, ibidi, Cat. No. 81156) and incubated for 24 h before introducing N_3-(OEG-IBCAPE)$_4$ or N_3-(OEG-IBCAPE)$_4$-Cu. Live-cell confocal microscopy was used to assess the cellular uptake of the N_3-(OEG-IBCAPE)$_4$ or N_3-(OEG-IBCAPE)$_4$-Cu. HepG2 cells were incubated with N_3-(OEG-IBCAPE)$_4$ and N_3-(OEG-IBCAPE)$_4$-Cu at the same drug concentration (50 μg mL^{-1}). After incubation for 0-4 h, HepG2 cells were carefully washed with PBS and incubated with LysoTraker (in green) and CellROX (in red) for 40 min. After that, stained by Hochest-33342 (ThermoFisher Scientific H1399, with excitation/emission ~350⁄461 nm) for 5 min, followed the stain solution was removed and the cells were washed 2-3 times in PBS. Images were taken using a Leica TCS SP5 Confocal Microscope.

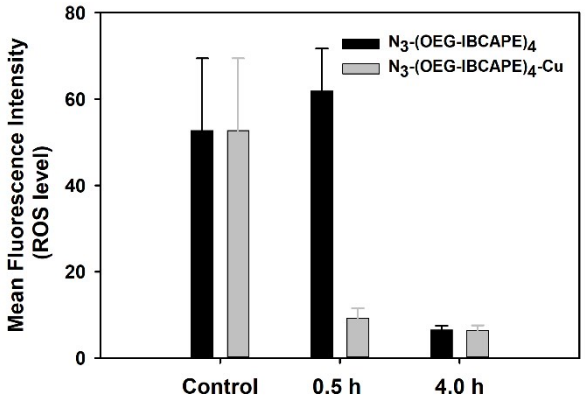
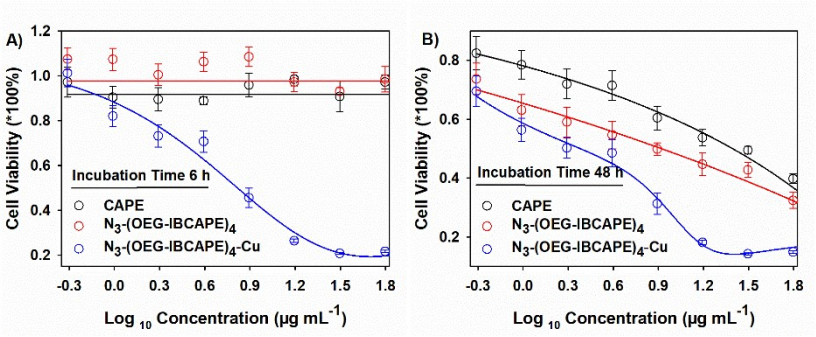


Figure S8. Quantitative comparison of ROS level in HepG2 cells after incubation with N_3-(OEG-IBCAPE)$_4$ or N_3-(OEG-IBCAPE)$_4$-Cu at the same prodrug concentration (50 μg mL^{-1}) at 37 °C for 0-4 h.

7. In Vitro Cytotoxicity Assay



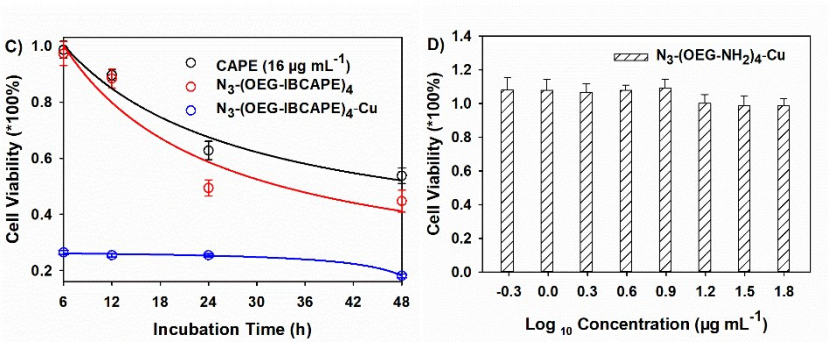


Figure S9. Viability of HepG2 cells treatment with various concentrations of CAPE, N_3-(OEG-IBCAPE)$_4$ and N_3-(OEG-IBCAPE)$_4$-Cu after 6 h (A) and 48 h (B) incubation determined by CCK-8 assay, respectively. (C) Viability of HepG2 cells treatment with free CAPE, N_3-(OEG-IBCAPE)$_4$ and N_3-(OEG-IBCAPE)$_4$-Cu at the same drug concentration (16 μg mL^{-1}) at 37 °C after different incubation times determined by CCK-8 assay. (D) Viability of HepG2 cells treated with various concentrations of N_3-(OEG-NH_2)$_4$-Cu after 48 h incubation determined by CCK-8 assay.

The CCK-8 assay was used to evaluate the cytotoxicity of CAPE (Free Drug), N_3-(OEG-IBCAPE)$_4$, N_3-(OEG-IBCAPE)$_4$-Cu and N_3-(OEG-NH_2)$_4$-Cu (as a negative control group). Cytotoxicity of specific time duration was also investigated to demonstrate the acceleration effect in the presence of Cu(II) ions. In all treatment groups, HepG2 cells were seeded in the 96-well plates with a density of 5 × 10^3 cells well^{-1}, cultured with RPMI-1640 medium with 10% FBS. After the cells sticking the bottom of the wells, medium in each well was discarded and replaced with series concentrations of CAPE (Free Drug), N_3-(OEG-IBCAPE)$_4$ and N_3-(OEG-IBCAPE)$_4$-Cu, respectively, at the same concentration of the free drug. After cultured for the specific time duration (6 h, 12 h, 24 h and 48 h), the mediums were discarded and the cells were washed with PBS for three times. Then, 10 μL CCK-8 reagent with 90 μL medium was added in each well. After 1 h culture duration, the cell viability was determined by plate reader under 450 nm.

8. References

[1] R. D. Cheng, G. Li, L. Fan, Z. T. Liu, Z. W. Liu, J. Q. Jiang, *J. Mater. Chem. B*. **2018**, *6*, 7800-7804.