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Electronic Supplementary Information for

ROS-responsive and self-accelerating drug release nanoplatform for overcoming multidrug resistance

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Materials

Poly(ethylene glycol) (M_w=2000, $mPEG_{2k}$), D-α-Tocopherol Succinate (α-TOS), N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N′ -ethylcarbodiimide hydrochloride (EDC·HCl), N,N'-Carbonyldiimidazole (CDI) and 4-(Dimethylamino)pyridine DMAP) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). 3-Mercaptopropionic acid was supplied by MACKLIN reagent (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Hvsf United Chemical Materials Co., Ltd. (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and RPMI 1640 were obtained from Dingguo Biotechnology Co., Ltd. (Tianjin, China). All other chemicals used were of analytical grade.

Cells and animals

MCF-7 and MCF-7/ADR cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL) in a humidified atmosphere of 5% CO_2 at 37°C. BALB/c nude mice (female, weight of 16-18 g) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animal experiments were performed in compliance with the guidelines of the Care and Use of Laboratory Animals and approved by Experiment Animal Administrative Committee of Institute of Radiation Medicine (Tianjin, China).

Characterizations

Synthetic intermediates and final prodrug were characterized by ^1H NMR spectra using an NMR spectrometer (Mercury Vx-300, USA). The size distribution of micelles was characterized by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern 3600, Worcestershire, U.K.) and the transmission electron microscope (TEM, HT7700, Hitachi, Japan) was utilized to investigate the morphology of particles.

Synthesis of Thioketal Linker (TK)

TK was synthesized as described previously.[1] Briefly, 3-mercaptopropionic acid (8.54 mL) and anhydrous acetone (14.66 mL) were added into a round-bottomed flask and saturated with anhydrous HCl gas for an hour. The reaction was stopped and chilled at -20°C until crystallization was complete. After filtration, the crude crystals were washed several times with cold water and hexane until white crystals were obtained. The white crystals were further dried in a vacuum desiccator.

Synthesis of $mPEG_{2k}$-CDI

$mPEG_{2k}$-OH (5 g, 2.5 mmol) and CDI (1.62 g, 10 mmol) were dissolved in anhydrous -dichloromethane (DCM, 20 mL) and stirred under nitrogen atmosphere at room temperature overnight. The excess DCM was removed through rotary evaporation and the condensed solution

was dropwise added to diethyl ether to precipitate $mPEG_{2k}$-CDI. This process was repeated for three times to thoroughly remove the excess of CDI. The obtained $mPEG_{2k}$-CDI was further dried in a vacuum desiccator.

Synthesis of $mPEG_{2k}$-NH_2

$mPEG_{2k}$-CDI (2g, 1 mmol) was dissolved in anhydrous DCM (5 mL) and added dropwise to 1.34 mL of ethylenediamine. The reaction was stirred at room temperature overnight. The excess of ethylenediamine was removed by rotary evaporation and the concentrates were added dropwise to diethyl ether to precipitate crude $mPEG_{2k}$-NH_2. This process was repeated three times and the obtained product was further purified through dialysis against distilled water for 24 h.

Synthesis of $mPEG_{2k}$-TK

$mPEG_{2k}$-NH_2 (400 mg, 0.2mmol), TK (505 mg, 2 mmol), EDC·HCl (115 mg, 0.6 mmol) and NHS (69 mg, 0.6 mmol) were dissolved in 4 mL of anhydrous DMF. The reaction was performed under nitrogen atmosphere for 24 h at room temperature. The resultant solution was dropwise added to diethyl ether to precipitate mPEG2k-TK. The product was further purified by extensively dialyzing against deionized water to remove EDC·HCl, NHS and the remaining TK. $mPEG_{2k}$-TK was obtained as white powder after lyophilization.

Synthesis of $mPEG_{2k}$-TK-CDI

$mPEG_{2k}$-TK (350 mg, 0.156 mmol) and CDI (252.2 mg, 1.56 mmol) were dissolved in 3 mL of anhydrous DCM and stirred under nitrogen atmosphere at room temperature overnight. The excess DCM was removed through rotary evaporation and the condensed solution was dropwise added to diethyl ether to precipitate $mPEG_{2k}$-TK-CDI. This process was repeated three times to thoroughly remove the excess of CDI. The obtained $mPEG_{2k}$-TK-CDI was further dried in a vacuum desiccator.

Synthesis of $mPEG_{2k}$-TK-hyd

$mPEG_{2k}$-TK-CDI (300 mg, 0.13 mmol) was dissolved in 2 mL of anhydrous DCM and added dropwise to hydrazine monohydrate (130.6 mg, 2.6 mmol). The reaction was performed at room temperature overnight. The resultant solution was added dropwise to diethyl ether to precipitate crude $mPEG_{2k}$-TK-hyd product. The product was further purified by dialyzing against deionized water for 24 h and $mPEG_{2k}$-TK-hyd was obtained after lyophilization.

Synthesis of $mPEG_{2k}$-TK-TOS

$mPEG_{2k}$-TK-hyd (200 mg, 0.085 mmol), α-TOS (90.3 mg, 0.17 mmol), EDC·HCl (65.3 mg, 0.34 mmol) and NHS (39.2 mg, 0.34 mmol) were dissolved in 3 mL of anhydrous DMF. The reaction was stirred in a nitrogen atmosphere at room temperature for 24 h. The resultant solution was added dropwise to diethyl ether to precipitate $mPEG_{2k}$-TK-TOS and this process was repeated three times to extensively remove the excess of α-TOS. The obtained white solid product was further purified by dialyzing against deionized water to eliminate the remaining EDC·HCl and NHS. $mPEG_{2k}$-TK-TOS was obtained after lyophilization.

Preparation of Blank and DOX Loaded Micelles

$mPEG_{2k}$-TK-TOS/(α-TOS+DOX) (PTKTD), $mPEG_{2k}$-TK-TOS/α-TOS (PTKT) drug loaded micelles and $mPEG_{2k}$-TK-TOS (PTK) blank micelles were prepared through thin-film hydration method.[2] The PTKTD micelles were prepared as previously with slight modifications. DOX·HCl (10 mg) was firstly dissolved in 4 mL of anhydrous methanol and stirred at room temperature for 3 h to eliminate HCl. Then the DOX solution was mixed with $mPEG_{2k}$-TK-TOS and α-TOS in chloroform in a ratio of 0.35:2:1 (DOX:$mPEG_{2k}$-TK-TOS:α-TOS in weight ratios) and the resultant solution was sonicated for 2 min. The organic solvent was removed by rotary evaporation and the obtained film was further dried in the vacuum desiccator for another 2 h. The film was then hydrated

with deionized water at 45°C for 1 h. The solution was filtered through 0.22 μm syringe filter to eliminate the unloaded DOX and α-TOS. The PTKT and blank PTK micelles were prepared with similar procedure as mentioned above. DOX encapsulation efficiency (EE%) and drug loading content (DL%) was calculated by the following equations.

DL%= $(weight\ of\ loaded\ drug)/(weight\ of\ drug\ loaded\ micelle) \times 100\%$

EE%= $(weight\ of\ loaded\ drug)/(weight\ of\ drug\ in\ feed) \times 100\%$

Computational methods

Density functional theory (DFT) calculations were carried out using Gaussian 16 program. In our computational study, we replaced the alkyl chain in α-TOS with a methyl group (defined as α-TOS1), since such simplification could not affect our discussion on noncovalent interactions. B3LYP-D3 functional methods together with 6-31G* basis set were used for geometry optimizations of DOX and α-TOS1 in the gas phase. The frequency calculations were employed at the same level theory to confirm all the optimized structures to be local minima (no imaginary frequency).

ROS sensitivity of $mPEG_{2k}$-TK-TOS

The ROS responsiveness of $mPEG_{2k}$-TK-TOS was investigated by ^1H NMR. In brief, 8 mg of mPEG2k-TK-TOS was dissolved in 2 mL of aqueous solution containing 10 mM H_2O_2 and 3.2 μM $CuCl_2$ and incubated at 37°C for 24 h. Then the obtained solution was lyophilized and characterized by ^1H NMR.

***In vitro* drug release**

The *in vitro* drug release profile of PTKTD micelles was investigated through a dialysis method in phosphate buffer (PBS) with different concentrations of H_2O_2: PBS (pH 7.4), PBS (pH 7.4) with 0.1 mM H_2O_2, PBS (pH 7.4) with 1 mM H_2O_2. In brief, 2 mg of PTKTD was incubated in 25 mL release medium at 37°C. At predetermined intervals, an aliquot of 0.5 mL solution was withdrawn and replaced with equivalent volume of fresh medium. The amount of released DOX was determined using a fluorescence spectrophotometer at Ex/Em 480/525 nm.

***In vitro* cytotoxicity**

MTT assay was performed to evaluate the cytotoxicity of micelles against tumor cells. Briefly, MCF-7 and MCF-7/ADR cells were seeded at a density of 5×10^3 cells/well in 96-well cell culture plate and cultured for 24 h to allow cell attachment and stabilization. The cells were incubated with DOX, PTK, PTKT and PTKTD micelles at DOX equivalent concentrations ranging from 1 to 15 μg/mL. After incubating for 48 h, 10 μL MTT solution (5 mg/mL) was added and incubated for another 4 h. Then the medium was replaced with 150 μL DMSO to dissolve the formed purple formazan crystals. The absorbance at 570 nm of each well was determined and cell viability was calculated by the following formula:

Cell viability=Abs (sample)/Abs (control) $\times$ 100%

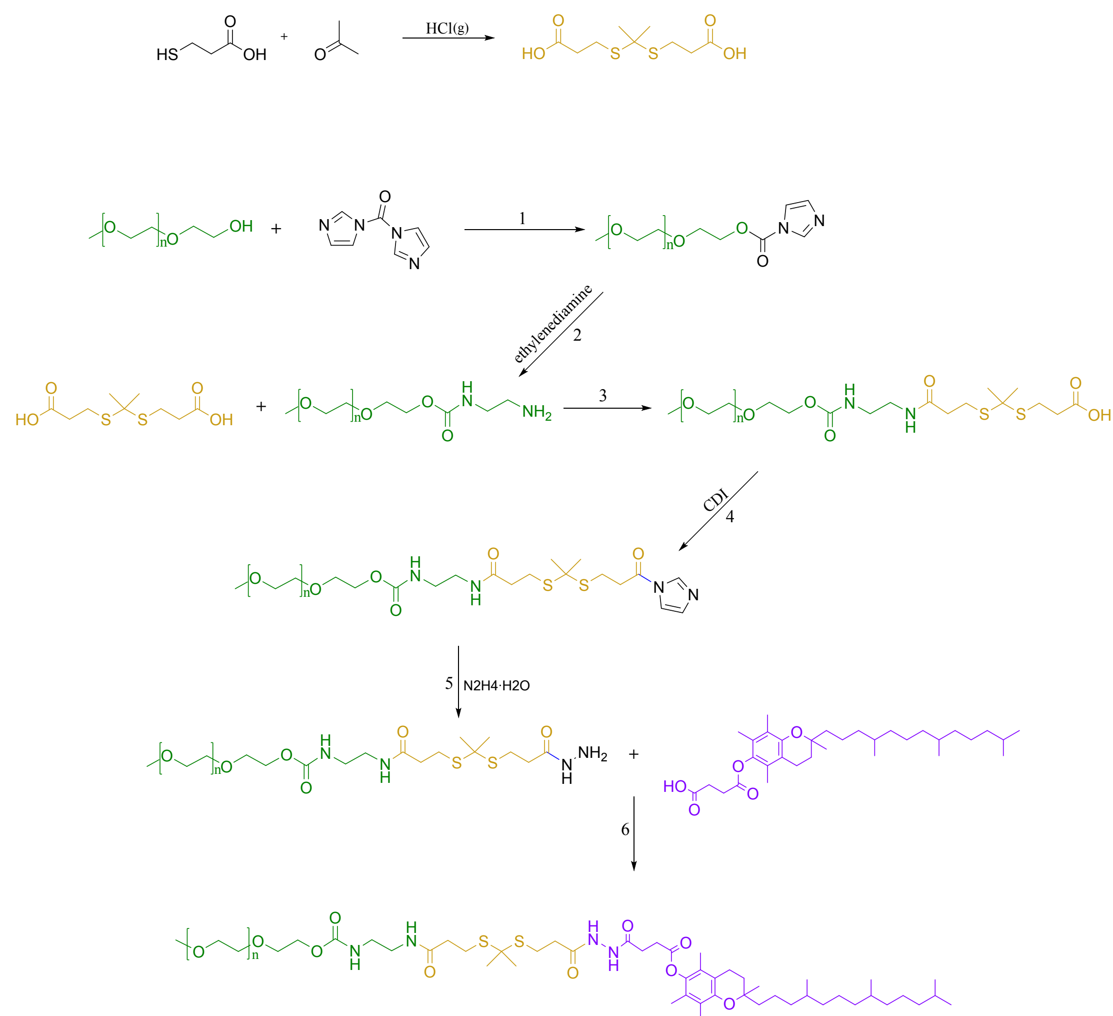
ROS production

The intracellular ROS concentration was determined as described previously with some modifications.[3] Dichlorofluorescindiacetate (DCFA-DA) was used as a fluorogenic dye to monitor intracellular ROS. Briefly, MCF-7/ADR cells were seeded in 96-well plates (1×10^4 cells per well) and cultured for 24 h. Then, the culture medium was replaced with medium containing free DOX, PTK, PTKD, PTKT and PTKTD micelles. After 5 h, DCFH-DA solution (10 μM) in a serum-free RPMI 1640 medium was added and incubated for 30 min. Then, the cells were rinsed with serum-free RPIM 1640 medium and analyzed using fluorescence spectrometer.

***In Vivo* Antitumor Study and Histochemistry Analysis**

The *in vivo* antitumor effect of different micelles was performed on MCF-7/ADR tumor-bearing nude mice model. MCF-7/ADR cells (1×10^7cells) were subcutaneously injected into the right

armpit of BALB/c nude mice (female) to generate tumor bearing mice. When the tumor volume reached ~80 mm^3, tumor bearing mice were randomly divided into four groups (n=6) and intravenously injected with saline, DOX, PTKT and PTKTD solutions at a DOX equivalent dose of 5 mg/kg every 3 days. The body weight and tumor volume were measured every 2 days. Tumor volume was calculated according to the following formula: $V=L \times W^2/2$, where L and W represent longest and shortest diameter respectively. After 14 days treatment, all mice were sacrificed and main organs (heart, liver, spleen, lung and kidney) with tumors were collected for immunohistochemistry analysis.



Scheme S1 Synthetic routes of TK and mPEG$_{2k}$-TK-TOS

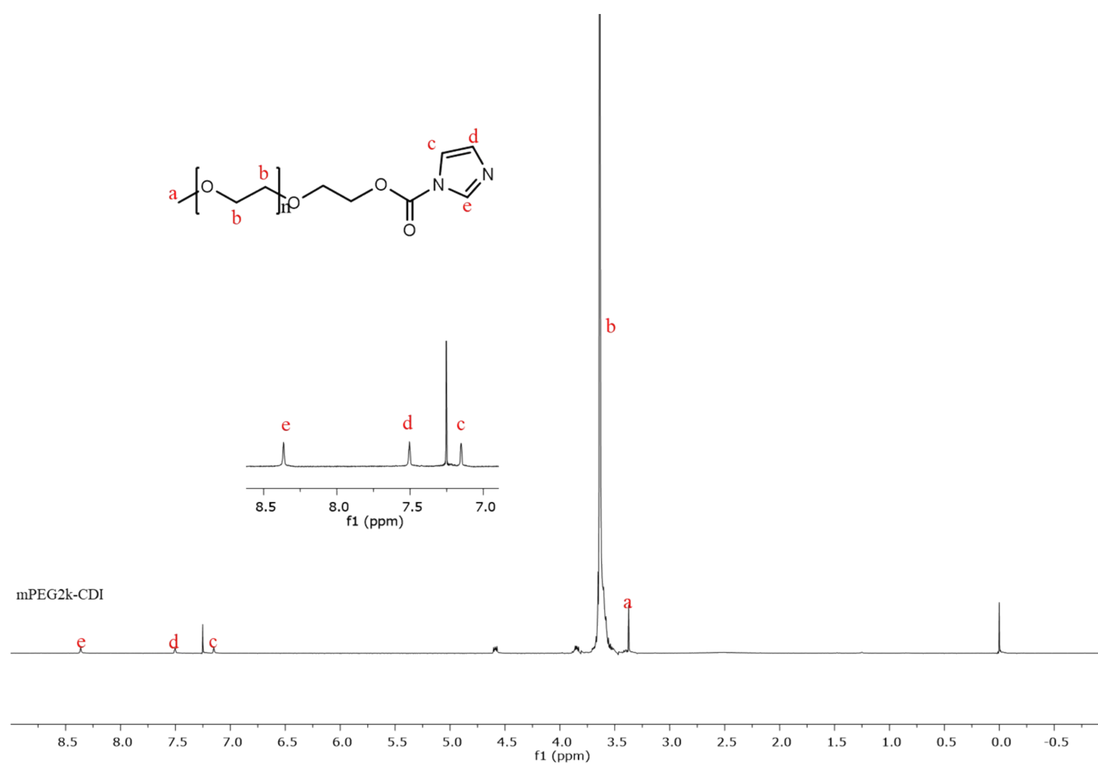


Fig. S1 ^{1}H NMR spectra of $mPEG_{2k}$-CDI

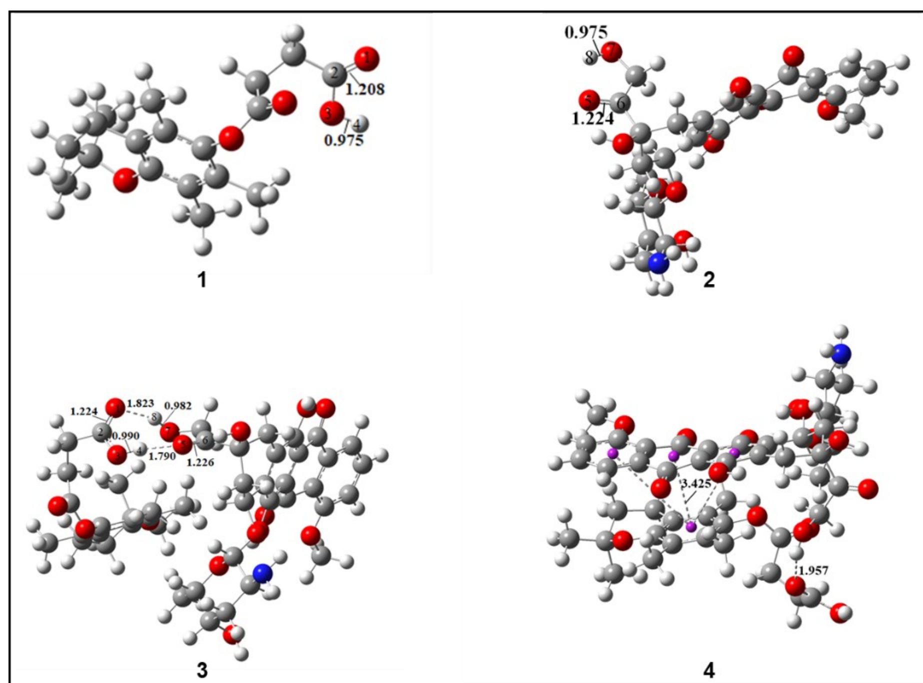


Fig. S2 Noncovalent intermolecular interactions analyzed by a density functional theory method. (1. α-TOS1, 2. DOX, 3. hydrogen bonding interactions between 1 and 2, 4. π-π stacking interactions between 1 and 2).

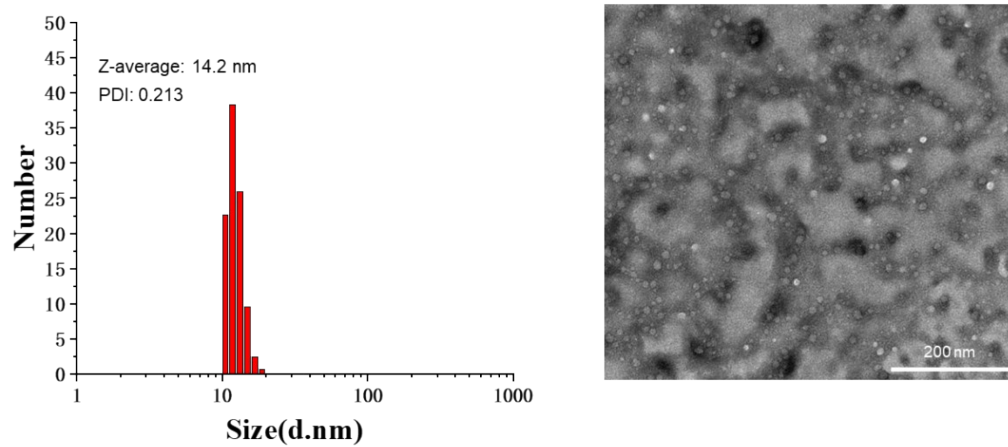
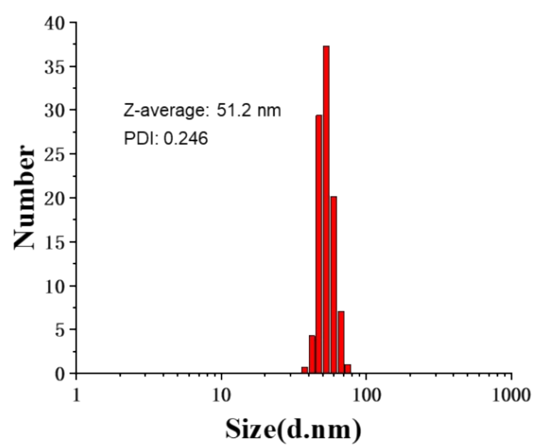


Fig. S3 DLS analysis and TEM image of PTK micelles. Scale bar: 200 nm.



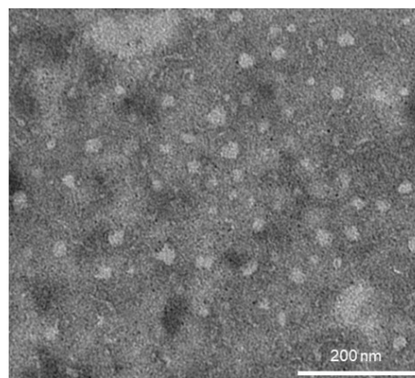


Fig. S4 DLS analysis and TEM image of PTKT micelles. Scale bar: 200 nm.

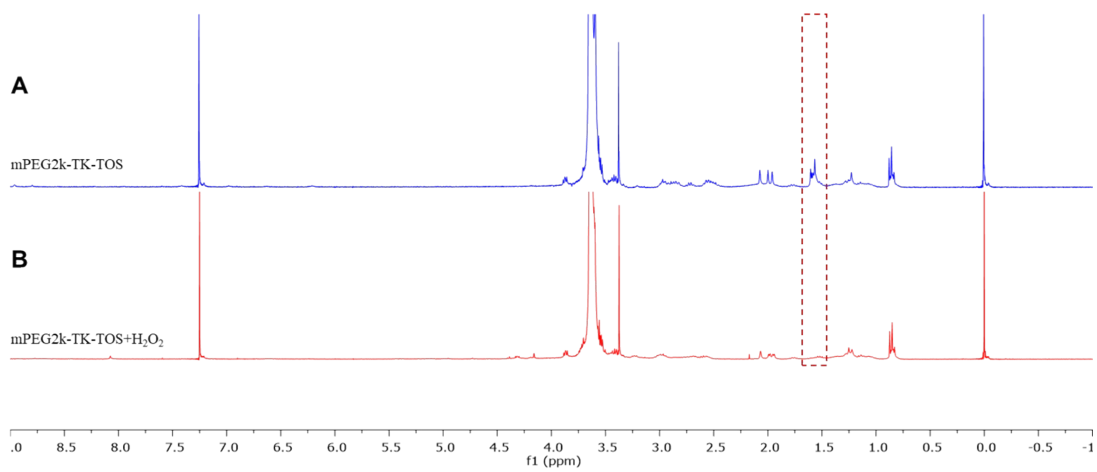


Fig. S5 ^{1}H NMR spectra of mPEG$_{2k}$-TK-TOS with and without H_2O_2.

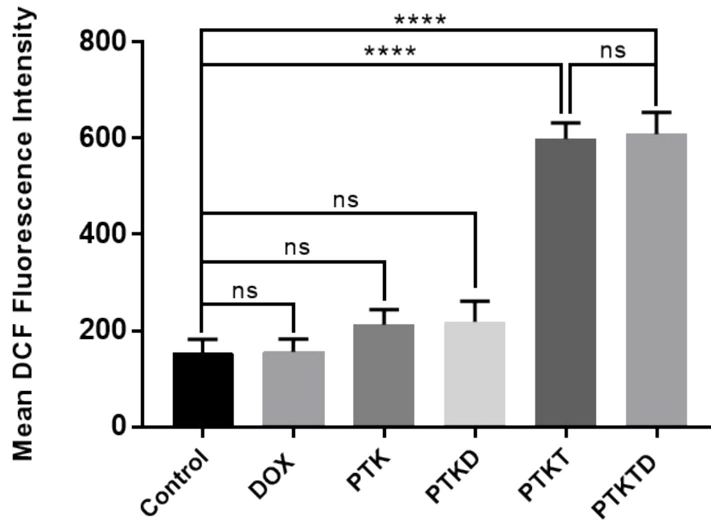


Fig. S6 Relative fluorescence intensity of DCF measured using fluorescence spectrometer after MCF-7/ADR cells were exposed to DOX, PTK, PTKD, PTKT and PTKTD micelles, ****$P < 0.0001$.

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