Supporting Information

H_{2}O_{2}-responsive Nano-Prodrug for Podophyllotoxin Delivery

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Supplementary Methods

Materials.

Podophyllotoxin (98%) was purchased from Shanghai Yuanye Bio-Technology Co. Ltd. (China). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-2000] (DSPE-mPEG2000) was purchased from Hunan Huateng Pharmaceutical Co., Ltd. (China). Dichloromethane (DCM; 99%), triethylamine (99.5%) and molecular sieves (4A, 0.02-0.03in beads) were purchased from Tianjinzhuyuan chemical reagent Co., Ltd (Tianjin, China). Poly(ethylene glycol) (n) monomethacrylate (PEGMA; $M_n = 500$), dimethyl sulfoxide (DMSO; 99.5%), DMSO-d$_6$ (99.9 atom% D, containing 1% (v/v) TMS), oxalyl chloride (99%), methanol (MeOH; 99.9%), Sephadex LH-20, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Coumarin 6, and 4',6-diamidino-2-phenylindole (DAPI) were provided by Sigma-Aldrich, USA. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) was purchased from Thermo Fisher Scientific Inc. RPMI 1640 (Roswell Park Memorial Institute), DMEM (Dulbecco's modified Eagle's medium), fetal bovine serum (FBS), 0.25% trypsin-EDTA and penicillin/streptomycin were available from Gibco BRL Co., Ltd. TRITC Phalloidin (Phalloidin-Tetramethylrhodamine Conjugate) was obtained from Yeasen biotech Co., Ltd. (Shanghai, China). Reactive Oxygen Species (ROS) Assay Kit (2',7'-Dichlorofluorescin diacetate, DCFH-DA) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Mouse embryonal fibroblast NIH 3T3 cells and CT26 (murine colon cells) cells were obtained from Sun Yat-sen University. The male mice
(18–22 g, BALB/c) and the male Sprague-Dawley (SD) rats (6~8 weeks old, 180~220 g) were obtained from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). All animal procedures were followed as the guidelines of the Principles of Laboratory Animal Care and Use at Sun Yat-sen University, and were approved by the Animal Ethics Committee of Sun Yat-sen University.

**Measurements.**

The NMR spectra was recorded using a nuclear magnetic resonance spectrometer (Bruker Ascend TM 500, Germany), DMSO-$d_6$ as a solvent and tetramethylsilane as an internal standard. The molecular weight of POD-PEG was measured by a liquid chromatography-mass spectrometer (LC-MS) equipped with an Agilent 6120. The particle size and polydispersity index of POD-PEG NPs were measured by dynamic light scattering (DLS) at room temperature on a Zetasizer Nano-ZS90 (Malvern, UK). The content of POD-PEG in the nanoparticles solution was estimated from the absorbance at 325 nm with a UV-vis spectrophotometer (DU800, Beckman Coulter, USA). The morphology of the POD-PEG NPs was observed by a TEM (JEOL JEM-1400plus, Japan) with an acceleration voltage of 200 kV. The content of POD in the release medium was determined by high performance liquid chromatography (HPLC). The detection conditions were as follows: mobile phase with 60/40 methanol/water with 0.1% phosphoric acid (v/v), flow rate of 1.0 mL/min, column temperature of 25°C, and 292 nm of UV-vis wavelength.

**Measurement of drug Contents in POD-PEG NPs.**

The POD-PEG NPs were washed three times with PBS (10 mM, pH 7.4) using centrifugal ultrafiltration so as to remove residual DMSO and free drug. The concentration of POD-PEG in the nanoparticles solution was estimated from the absorbance at 325 nm, in the UV-vis spectra, after ultrasonically demulsifying the POD-PEG from the POD-PEG NPs solution into methanol. Another identical aliquot of the POD-PEG NPs solution was lyophilized to obtain a total mass of the nanoparticles. The
drug loading (DL) and the drug entrapment efficiency (EE) were calculated using the following formulas:

\[
DL (\%) = \frac{\text{drug content in nanoparticles}}{\text{mass of nanoparticles}} \times 100\\%
\]

\[
EE (\%) = \frac{\text{drug content in nanoparticles}}{\text{drug feeding}} \times 100\\%
\]

**In vitro stability of POD-PEG NPs.**

To assess the *in vitro* stability of POD-PEG NPs, POD-PEG NPs (0.5 mg/mL) solution was dispersed into 10% FBS in PBS buffer (pH 7.4, 10 mM) and incubated in a shaker (100 rpm, 37°C) in triplicate. The particle size of POD-PEG NPs was monitored by DLS at the time points of 0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 48 h. Besides, the POD-PEG NPs was dispersed into PBS (pH 7.4, 10 mM) with 1 mM H\(_2\)O\(_2\). The morphology and size change of the nanoparticles after 12 h of H\(_2\)O\(_2\)-treatment was observed by the TEM and DLS, respectively.

**Cell culture**

CT26 cells and NIH 3T3 cells were incubated with RPMI-1640 and DMEM medium both containing 10% (v/v) FBS and 1% (w/v) penicillin-streptomycin, respectively, at 37°C in a humidified 5% CO\(_2\) atmosphere. The cells were all propagated in T-25 flasks and fresh complete medium was replaced every day.
Figure S1. $^1$H-NMR spectra of POD-PEG in DMSO-$d_6$. 
Figure S2. ESI-MS results of POD-PEG in MeOH.

![Figure S2](image)

Figure S3. (A) Zeta potential of POD-PEG NPs. (B) Changes of average particle size for POD-PEG NPs incubated in PBS (pH 7.4, 10 mM) containing 10% FBS (n = 3).

![Figure S3](image)

Figure S4. (A) The change of particles size of POD-PEG NPs dispersed into PBS (pH 7.4, 10 mM) with 1 mM H₂O₂. The size change of POD-PEG NPs was determined by DLS. (B) TEM images of POD-PEG NPs after incubation in H₂O₂ solution (1 mM) (scale bar = 100 nm).

![Figure S4](image)
Figure S5. (A) The cellular uptake efficiency of C6-loaded POD-PEG NPs in CT26 cells observed by CLSM. Quantified data pertaining to intracellular C6 were summarized in the histogram. (B) The free POD or POD-PEG NPs were incubated with CT26 cells for 12 h, while the ROS kit was used to detect intracellular ROS production. Quantified data pertaining to intracellular ROS were summarized in the histogram. Data are shown as mean ± SD (n = 3); ***, p < 0.001.

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<th>PBS</th>
<th>POD (mg/kg)</th>
<th>POD-PEG NPs (mg/kg)</th>
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Figure S6. Representative photomicrographs of the H&E stained major organs and tumor sections of mice treated PBS, Free POD and POD-PEG NPs (at POD-equivalent dose of 2.5, 5 and 10 mg/kg), respectively (scale bar = 100 μm).