Electronic Supplementary Material (ESI) for RSC Advances.

Nitric oxide/paclitaxel micelles enhance anti-liver cancer effects and paclitaxel sensitivity by inducing ferroptosis, endoplasmic reticulum stress and pyroptosis

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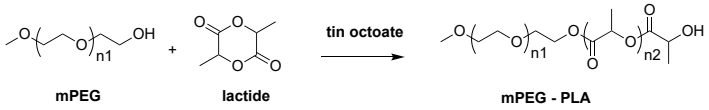
Supporting information

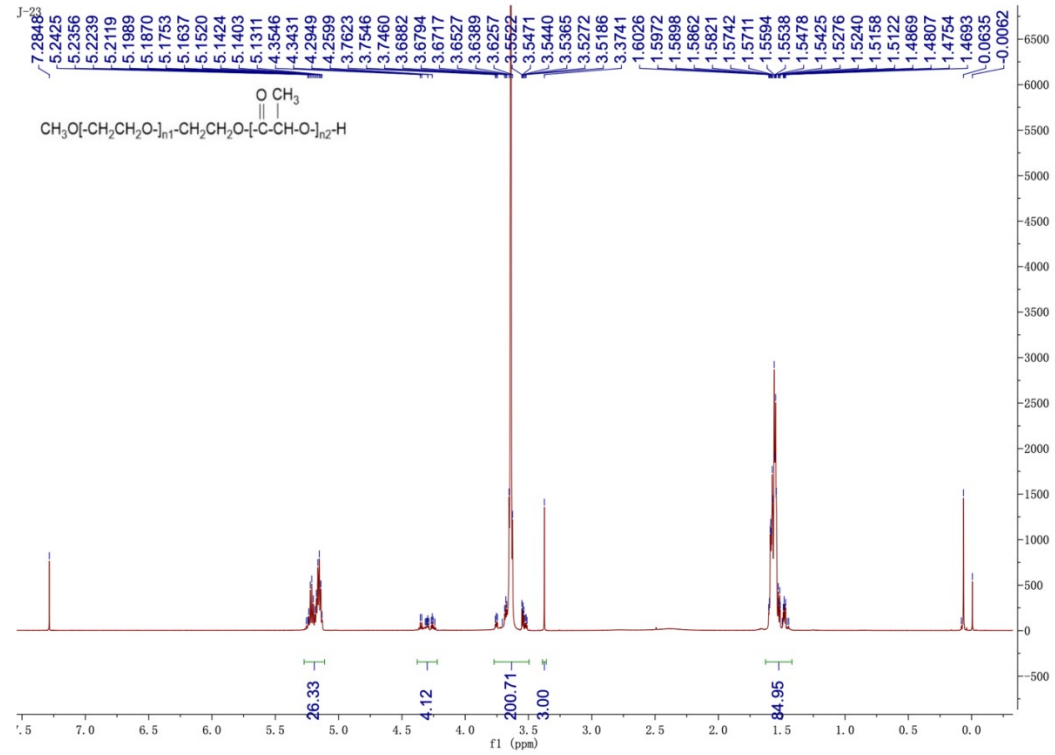
1. Synthetic procedure of paclitaxel-loaded micelles NO/PTX
2. Experimental procedure for the biological test
3. Western blots of proteins

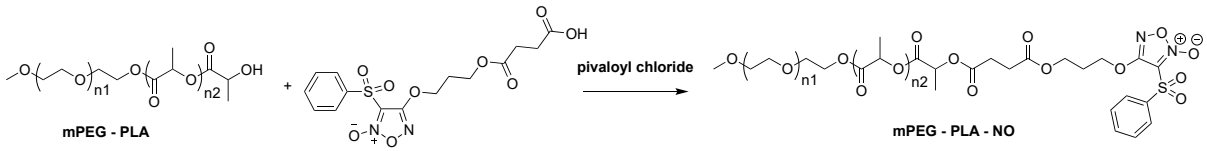
1. Synthetic procedure of paclitaxel-loaded micelles NO/PTX



1.1 Synthesis of mPEG-PLA

To a vacuum dried mPEG 2000 (30.0 g) at 130ºC in flask was added *dl*-lactide (30.6 g, 0.212 mol) and tin (II) octoate (121 mg, 0.299 mmol). The mixture was sealed and stirred at 130ºC for 18 h. After cooling down to room temperature, the resulting crude product was dissolved in dichloromethane (50 mL), and a cold absolute ethanol (300 mL) was added. The precipitate was collected by filter and dried in vacuum to afford the polymer mPEG-PLA (56.2 g). ^{1}H-NMR (600 MHz, $CDCl_3$) δ (ppm): 5.24-5.13 (m, 27H), 4.35-4.26 (m, 4H), 3.76-3.52 (m, 200H), 3.37 (s, 3H), 1.60-1.47 (m, 81H).

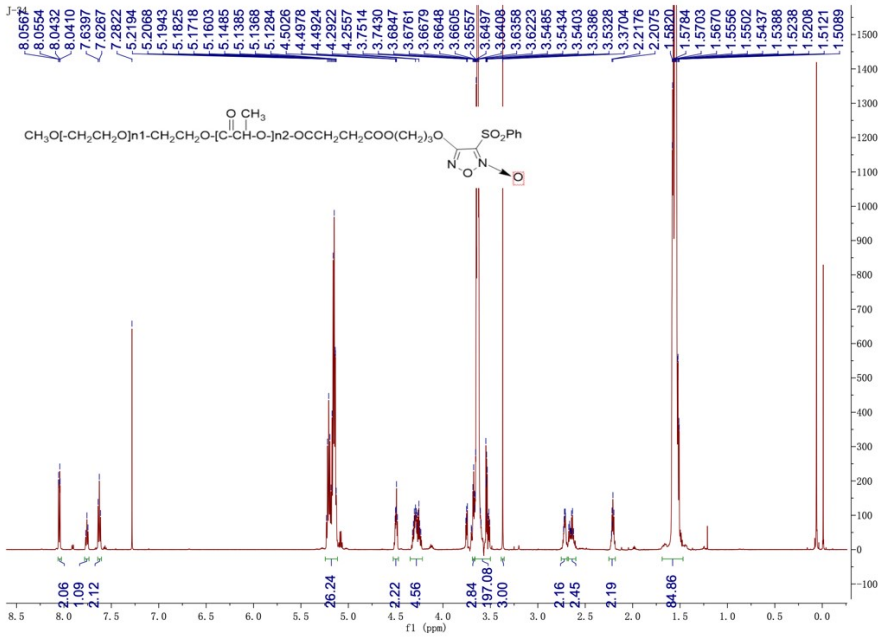




1.2 Synthesis of mPEG-PLA-NO

To the solution of furoxan compound (3.0 g, 7.49 mmol) in ethyl acetate (30 mL) were added triethyl amine (1.73 mL, 12.4 mmol) and pivaloyl chloride (1.11 mL, 9.07 mmol) at -10ºC. The

reaction mixture was then stirred at 0ºC for 2 h and filtered to remove undissolved salt. The filtrate was evaporated to give a liquid as mixed anhydride. To the solution of mPEG-PLA (12.6 g) in dichloromethane (63 mL) was added pyridine (1 mL, 12.4 mmol) and DMAP (146 mg, 1.20 mmol). A solution of anhydride in dichloromethane (15 mL) was added to the mixture at 0ºC. The resulting reaction mixture was stirred at this temperature for 2 h, kept at room temperature for 36 h, and poured into cold ethanol. The precipitate was collected by filtration and dried to afford the polymer mPEG-PLA-NO (12.8 g). ^{1}H-NMR (600 MHz, $CDCl_3$) δ (ppm): 8.06-7.62 (m, 5H), 5.22-5.12 (m, 27H), 4.51-4.49 (m, 2H), 4.30-4.25 (m, 4H), 3.76-3.52 (m, 200H), 3.37 (s, 3H), 2.75-2.55 (m, 4H), 2.22-2.20 (m, 2H), 1.59-1.50 (m, 81H).



1.3 Preparation of paclitaxel-loaded micelle (NO/PTX)

The polymer mPEG-PLA-NO (50 mg) and surfactant HS15 (50 mg) was dissolved in ethanol (5 mL). PTX (10 mg) was dissolved completely in the solution after stirring. Mannitol solution (5%, 1.9 mL) was added to form the aqueous micelle. The clear solution was lyophilized to afford the micellar PTX (NO/PTX).

The average diameter of NO/PTX micelles, determined by dynamic light scattering (DLS), was less than 50 nm. Transmission electron microscopy investigation showed that the micelles were spherical and had sizes consistent with that of DLS measurements. The entrapment efficiency (EE%) of NO/PTX was determined to be 97.7 ± 0.5% using an ultrafiltration method by separating the non-entrapped drug from nanoparticles. EE% = $(C_{total} - C_{free})/ C_{total}$.

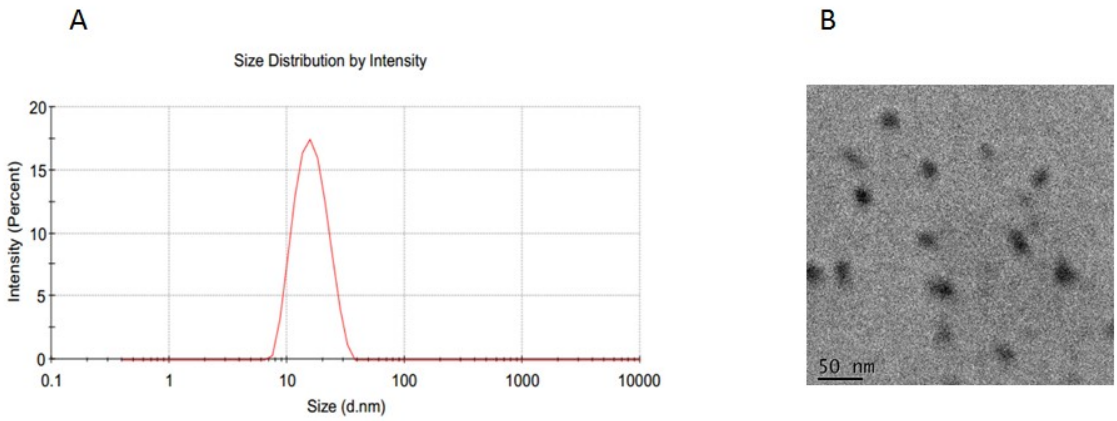


Figure (**A**) The size distribution by DLS of NO/PTX micelles; (**B**) TEM micrograph of NO/PTX micelles.

2. Experimental procedure for the biological test

2.1 *In vitro* cytotoxicity

The HCC cell line Bel-7402 was used for the *in vitro* cytotoxicity study. Firstly, the antiproliferative effect of PTX (positive control) and NO/PTX were assessed based on 10-fold dilutions ranging from 0.0001 up to 10 μg/mL using Cell Counting Kit-8 (CCK8) cytotoxicity detection kits (Shanghai Bestbio Biotechnology Co., Ltd., Shanghai, China). Furthermore, The cytotoxicity of NO/PTX combined with ferroptosis inhibitor ferrostatin-1 (Fer-1, 5 μM) or ROS inhibitor N-acetyl cysteine (NAC, 5 mM) were also tested against Bel-7402 cells.

Briefly, the cells were counted and plated in 96-well plates at a density of 8×10^3 cells per well for 12 h and then treated with each drug concentration of PTX or NO/PTX in triplicate. Following 48 h of continuous drug exposure, 10 μL of CCK-8 solution were added to each well. After 2 h incubation, the OD values at 450 nm were measured by microplate reader (Spectra MAX I3; Molecular Devices, San Jose, CA, USA). All experiments were performed independently at least three times. SPSS 13.0 software (IBM, Armonk, NY, USA) was used to determine the 50% inhibitory concentration (IC_{50}).

2.2 Hoechst 33258 staining

After the cell density was adjusted to 4×10^4 cells/well, Bel-7402 cells were inoculated into 6-well plates in a 5% CO_2 incubator at 37°C. After 12 h, the cells were treated with PTX, NO/PTX, NO/PTX+Fer-1 or NO/PTX+NAC, with PTX concentrations of 0.1, 1 or 10 μg/mL for another

48 h. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min and washed with phosphate-buffered saline (PBS) three times. Afterwards, the cells were incubated with Hoechst 33258 dye (Beyotime Biotech, Shanghai, China) at 50 μL per well at room temperature for 10 min in the dark. Next, the cells were photographed by a fluorescence microscope, which showed the nuclei of apoptotic cells in bright blue.

2.3 *In vivo* tumor growth inhibitory assay

The frozen H22 tumor strains were removed from liquid nitrogen and placed in a 37°C water bath. After dissolution by quick shaking, they were transferred to a cleaning table, thoroughly cleaned with saline, centrifuged at 1,000 rpm for 5 min and resuspended in saline after the supernatant was discarded. The cell concentration was adjusted to 1×10^6 cells/mL and 0.2 mL of cell suspension was inoculated to the abdominal cavity of a KM mouse. The cells were passaged every 5–7 days for a total of three generations. The well growing tumor strains were extracted from mouse ascites aseptically via syringe and diluted to 1×10^7 cells/mL with saline.

Male KM mice were injected subcutaneously in the right flank with 0.2 mL of H22 cell suspension in saline to establish a tumor bearing mouse model. Treatments begun when tumors in the mice reached a volume of 50–300 mm^3, and this day was designated as day 0. PTX and NO/PTX were injected intraperitoneally at doses of 30 and 45 mg/kg body weight, respectively, on days 0, 3 and 6, with saline as control. The selection of doses was based on our previous assay of acute toxicity. On day 9, the mice were euthanatized and the tumors, livers, spleens and thymus glands were harvested and weighed.

2.4 Measurement of intracellular ROS

The intracellular ROS of Bel-7402 cells were determined with a DCFH-DA fluorescence probe (#D6883; Sigma, St. Louis, MO, USA). Bel-7402 cells were seeded in 6-well plates (1.6×10^6 per well) and incubated with PTX, NO/PTX or NO/PTX+Fer-1 (0.1 μg/mL for PTX) respectively for 48 h. The cells were rinsed twice with PBS (1 mL, pH = 7.4, 0.01 mol/L) and stained with 10 μM DCFH-DA for 30 min at 37°C in the dark. Then the medium was removed and PBS rinsed cells were imaged using a Leica fluorescent microscope (Model: DMI 300B, magnification ×200).

2.5 Analysis of antioxidant parameters

To determine the levels of antioxidant parameters, cell samples after treatment with PTX, NO/PTX or NO/PTX+Fer-1 (0.1 or 1 μg/mL) were homogenized and then centrifuged at 1,000

rpm at 4°C for 10 min to obtain the supernatant. The protein concentrations of samples from Bel-7402 cells were determined with BCA kits (ComWin Biotech Co., Ltd., Beijing, China). The levels of total superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) were determined with commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5 Western blot analysis

Cells after treatment with PTX or NO/PTX were washed with PBS, and the total protein was extracted from treated cells using RIPA buffer (Solarbio Life Sciences, Beijing, China), to which was added protease and phosphatase inhibitors, followed by a 30 min incubation on ice and centrifugation for 10 min at 4°C at 12000 *g*. Protein concentrations were determined by BCA assay. Nuclear protein was extracted using a nuclear protein and cytoplasmic protein extraction kit (Beyotime). Equal amounts of protein (30 μg) were subjected to 7.5% or 12.5% SDS-PAGE and transferred to PVDF membranes. After non-specific blocking with non-fat milk for 2 h at room temperature, the membranes were incubated overnight with the following primary antibodies: rabbit anti-GPX4, rabbit anti-caspase-1, rabbit IL-1β, rabbit anti-P-gp, rabbit anti-Cleaved Caspase-3, mouse anti-caspase-3 and rabbit anti-NF-κB, which were all obtained from Proteintech Group and diluted to 1:1000, and GAPDH (1:500000, Proteintech), rabbit anti-P-ERK1/2, rabbit anti-p-IRE1a (1:1000, CST), mouse anti-BCL-2, Rabbit Anti-GRP78 (1:1000, Affinity) and rabbit anti-β3-tublin (1:1000, ABCOM) at 4°C overnight. Subsequently, the membranes were washed with PBS for 30 min and incubated with an HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature. Finally, the blots were scanned using an infrared laser imaging system (Biored, Model: Universal hood) and quantified by densitometer software ImageJ2x.

2.6 Apoptosis assay

Cells were seeded into six-well culture dishes at a density of 4×10^5 cells/well and incubated with the indicated doses of PTX or NO/PTX for 48 h. The apoptosis rates were measured using an annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The apoptosis rates were analyzed by flow cytometry (FACScan, BD Biosciences).