Electronic Supplementary Material (ESI) for Biomaterials Science.
This journal is © The Royal Society of Chemistry 2018

Electronic supplementary information

Polymer micro/nanocarrier-assisted synergistic chemohormonal therapy for prostate cancer

Liang He,[a,b] Weiguo Xu,[b] Xiaoqing Wang,[a] Chunxi Wang,*[a] Jianxun Ding*[b] and Xuesi Chen[b]

[a]Department of Urology, the First Hospital of Jilin University, Changchun 130021, People's Republic of China.

E-mail: chunxi_wang@126.com

[b]Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, People's Republic of China.

E-mail: jxding@ciac.ac.cn

1. Experimental protocols

1.1. Synthesis of methoxy poly(ethylene glycol)-*block*-poly(L-glutamic acid) (mPEG-*b*-PLG)

mPEG-*b*-PLG was synthesized by the ring-opening polymerization (ROP) of γ-benzyl L-glutamate *N*-carboxyanhydride (BLG NCA) using amino-terminated methoxy poly(ethylene glycol) as the macroinitiator and subsequent deprotection of benzyl group. Firstly, dried $mPEG_{113}$-NH_2 (2.0 g, 0.4 mmol) and BLG NCA (1.7 g, 6.5 mmol) were dissolved in 15.0 mL of dried *N,N*-dimethylformamide (DMF) in a flame-dry flask. The polymerization was performed at 25 °C for three days. Then, the solution was precipitated into 150.0 mL of anhydrous ether. Finally, the obtained solid was dried under vacuum at room temperature. The degree of polymerization (DP) of BLG was determined to be 17 from proton nuclear magnetic resonance (^{1}H NMR) spectrum. Yield = 77.6%.

Subsequently, $mPEG_{113}$-*b*-PLG_{17} was prepared by removing the benzyl group from $mPEG_{113}$-*b*-$PBLG_{17}$. In detail, 1.0 g of $mPEG_{113}$-*b*-$PBLG_{17}$ was dissolved in 10.0 mL of dichloroacetic acid. Then, 3.0 mL of 33.3 wt.% HBr/HAc solution was added in the mixture. After stirred for 1 h at room temperature, the solution was precipitated into 100.0 mL of diethyl ether. The obtained product was dissolved in DMF, dialyzed against water with a dialysis membrane (molecular weight cut-off (MWCO) = 3,500 Da) for three days, and then lyophilized to obtain $mPEG_{113}$-*b*-PLG_{17}. Yield = 82.7%.

1.2. Preparation of mitoxantrone (MTO)-loaded micelle

The aqueous solution of mPEG-*b*-PLG was mixed with an aqueous solution of MTO (1.0 mg mL^{-1}) in phosphate-buffered saline (PBS) at pH 7.4 with the weight ratio of the block polymer to MTO at 3:1 at 4 °C for 12 h. Then, the solution was dialyzed (MWCO = 3500 Da) in Milli-Q water

condition for 12 h and subsequently lyophilized.

After that, the drug loading content (DLC) and drug loading efficiency (DLE) were further measured. In brief, 1.0 mg of PM/MTO was dissolved in 10.0 mL of DMF and stirred for 12 h at room temperature. Then, the MTO loaded in mPEG-*b*-PLG polyion complex (PIC) micelle was detected by an ultraviolet-visible (UV-Vis) spectrophotometry with λ_{abs} = 670 nm (UV-1800, Shimadzu, Kyoto, Japan). The DLC and DLE of PM/MTO were calculated by Equations S1 and S2, respectively.

$$\text{DLC (wt.\%)} = \frac{\text{Weight of Drug in Micelle}}{\text{Weight of Drug - Loaed Micelle}} \times 100\% \quad \text{S1}$$

$$\text{DLE (wt.\%)} = \frac{\text{Weight of Drug in Micelle}}{\text{Total Weight of Feeding Drug}} \times 100\% \quad \text{S2}$$

1.3. Characterizations of PM/MTO

The microimages of PM/MTO were taken using a JEW-1011 transmission electron microscopy (TEM; JEOL, Tokyo, Japan) with an accelerating voltage of 100.0 kV for the detection of morphology and apparent size. The nanoformulation size changes in PBS at pH 7.4 or PBS at different pHs were detected by dynamic light scattering (DLS) on a Wyatt QELS instrument (Wyatt Technology Corp., Santa Barbara, CA, USA).

1.4. *In vitro* MTO release

In vitro drug release of PM/MTO was studied using a dialysis method. Typically, 1.0 mg of freeze-dried PM/MTO solid was dissolved in 10.0 mL of PBS at different pHs, pH 5.5, 6.8, and 7.4, and then transferred into the end-sealed dialysis membrane (MWCO = 3500 Da). Subsequently, the filled dialysis bags were put into 150.0 mL beakers, 100.0 mL of

corresponding buffer was added rapidly, and the apparatus was finally placed into an oscillation box with continuous vibration of 70 revolutions per minute (rpm) at 37 °C to mimic the normal physiological microenvironment. At the predetermined time points, 2.0 mL of the external release medium in the beaker was taken out, and the equal volume of fresh medium was replenished into the container. The amount of released MTO was determined through standard curve method on an ultraviolet-visible (UV-Vis) spectrophotometry.

1.5. Cell protocols

RM-1 PCa cell line were cultured in complete high glucose Dulbecco's modified Eagle's medium (HG-DMEM) with 10% (*V*/*V*) fetal bovine serum (FBS), penicillin (50 IU mL^{-1}), and streptomycin (50 IU mL^{-1}) at 37 °C in 5% (*V*/*V*) carbon dioxide (CO_2).

1.6. Androgen-sensitive verification

To confirm the androgen-sensitive characteristics of murine PCa RM-1 cells, androgen receptor (AR) and testosterone (TEST) induced PCa cells *in vitro* proliferation experiments were carried out. As for the AR Western blot detection, the murine melanoma B16F10 cells were selected as a negative control to compare with the RM-1 cells. 1.0 million RM-1 cells and B16F10 cells were collected and cracked. Finally, AR and β-actin were detected by Western blot.

Furthermore, the TEST inducing experiment was performed by a methyl thiazolyl tetrazolium (MTT) assay. As well as the cytotoxicity assessment, 0.8×10^4 RM-1 cells were suspended and cultured in 96-well plates as aforementioned. Then, 15.63, 7.81, 3.90, and 1.95 μg mL^{-1} TEST was added, and incubated for 48 h. Finally, the cell proliferation rate was analyzed as MTT part. The data were detected by ELx808 microplate reader.

1.7. Intracellular MTO release

In order to confirm the controlled intracellular release of MTO from PM/MTO, the flow cytometry (FCM) was introduced. In detail, 3.0×10^5 of RM-1 cells suspended in 2.0 mL of HG-DMEM were seeded in each well of 6-well plates. After 24 h culture, the culture media were removed and filled with 2.0 mL of fresh HG-DMEM. Then, the cells of experimental group were co-incubated with MTO or PM/MTO at a final MTO concentration of 5.0 μg mL^{-1}, and cells of control group were co-incubated with PBS. After 2 h co-incubation, the cells were washed with PBS for five times. Then, all cells were digested by 1.0 mL of trypsin per well and suspended in PBS. The harvested cells were centrifuged at 3,000 rpm for 5 min. The supernatants were discarded and the bottom cells were washed with PBS. Finally, the cells were resuspended in 500.0 μL of PBS, and data was analyzed by a flow cytometer (λ_{ex} = 695 nm; Guava EasyCyte™ technologies Inc., Hayward, CA, USA).

Similarly as FCM detection, confocal laser scanning microscopy (CLSM) assays were performed to reveal the subcellular distribution of MTO. Typically, 1.5×10^5 RM-1 cells in 2.0 mL of HG-DMEM were seeded on glass coverslips in a 6-well plate. After culture for 24 h, the culture medium in 6-well plate was removed, and 2.0 mL of fresh HG-DMEM was replenished. Next, the cells were co-incubated with MTO or PM/MTO at a final MTO concentration of 5.0 μg mL^{-1}. The cells on glass sheet were further incubated for 2 h and then washed with PBS five times. After that, cells were fixed with 4% (*W/V*) PBS-buffered paraformaldehyde for 20 min. Finally, cell nuclei were dyed blue by 4′,6-diamidino-2-phenylindole (DAPI) for 3 min, and washed with PBS for another five times. Finally, the CLSM microimages were taken by a confocal laser scanning microscope (LSM 780, Carl Zeiss, Jena, Germany).

1.8. Cytotoxicity assessment

The cytotoxicity of MTO and PM/MTO was measured by a methyl thiazolyl tetrazolium (MTT) assay. Following our previous protocol,[1] 0.8×10^4 RM-1 cells were suspended in 200.0 μL of complete HG-DMEM, planted into each well of 96-well plates and incubated for 24 h, then the cells were washed by sterile PBS. Then, 180.0 μL of fresh complete HG-DMEM was added. In addition, 20.0 μL of PBS containing MTO and PM/MTO with concentrations of MTO from 5.0×10^{-4} to 10.0 μg mL^{-1} were added to the experimental groups. Simultaneously, 20.0 μL of blank PBS was added to the control group. The cells were subjected to 20.0 μL of MTT at a concentration of 5.0 μg mL^{-1} after being incubated for 24 h. After that, the cells were incubated for another 4 h in incubator. The culture medium was carefully removed, and 150.0 μL of dimethyl sulfoxide (DMSO) per well was added to dissolve the MTT formazan generated by live cells. Finally, the plates were shocked for 3 min, and the absorbance of the medium was detected at 490 nm by an ELx808™ microplate reader (Bio-Tek® Instruments, Inc., Winooski, VT, USA). The cell viability was calculated by Equation S3.

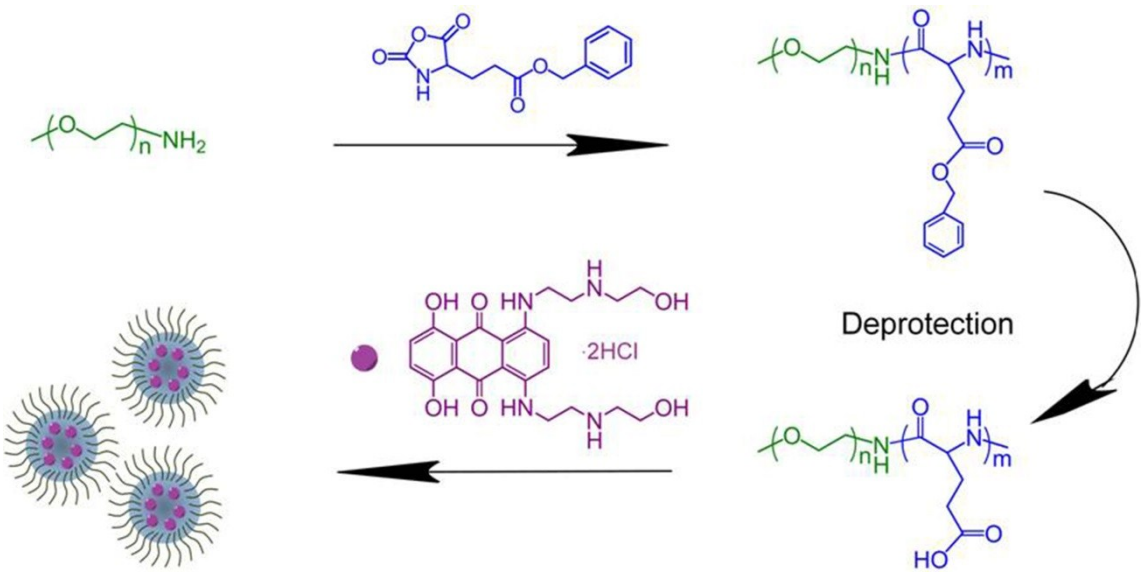
$$\text{Cell Viability } (\%) = \frac{A_{sample}}{A_{control}} \times 100\% \qquad \text{S3}$$

References

1 K. Zhao, D. Li, W. Xu, J. Ding, W. Jiang, M. Li, C. Wang and X. Chen, *Biomaterials*, 2017, 116, 82–94.

Table S1 Johnsen score rating scale

Score	Histological Finding
10	Full spermatogenesis with many spermatozoa, regular layers of germ cells
9	Many spermatozoa, irregular layers of germ cells, germ cells sloughed into the lumens
8	Few spermatozoa
7	No spermatozoa, many spermatids
6	No spermatozoa, few spermatids
5	No spermatozoa and spermatids, many spermatocytes
4	No spermatozoa and spermatids, few spermatocytes
3	Spermatogonia only
2	No germ cells, sertoli cells only
1	No seminiferous epithelial cells, tubular hyaline



Scheme S1 Synthesis pathway of $mPEG_{113}$-*b*-PLG_{17} and illustration for PM/MTO preparation.

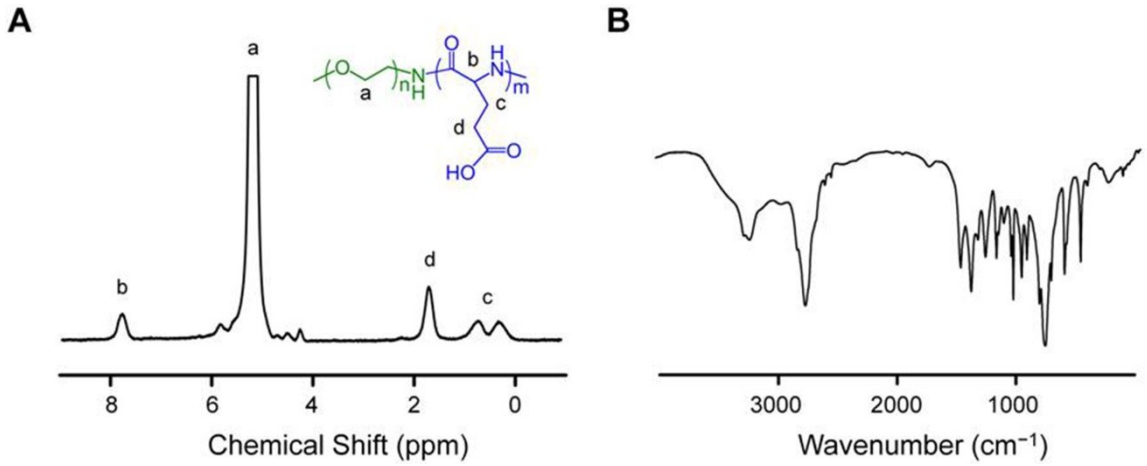


Fig. S1 Characterization of mPEG-*b*-PLG. (A) ^{1}HNMR and (B) FT-IR spectra of mPEG-*b*-PLG.

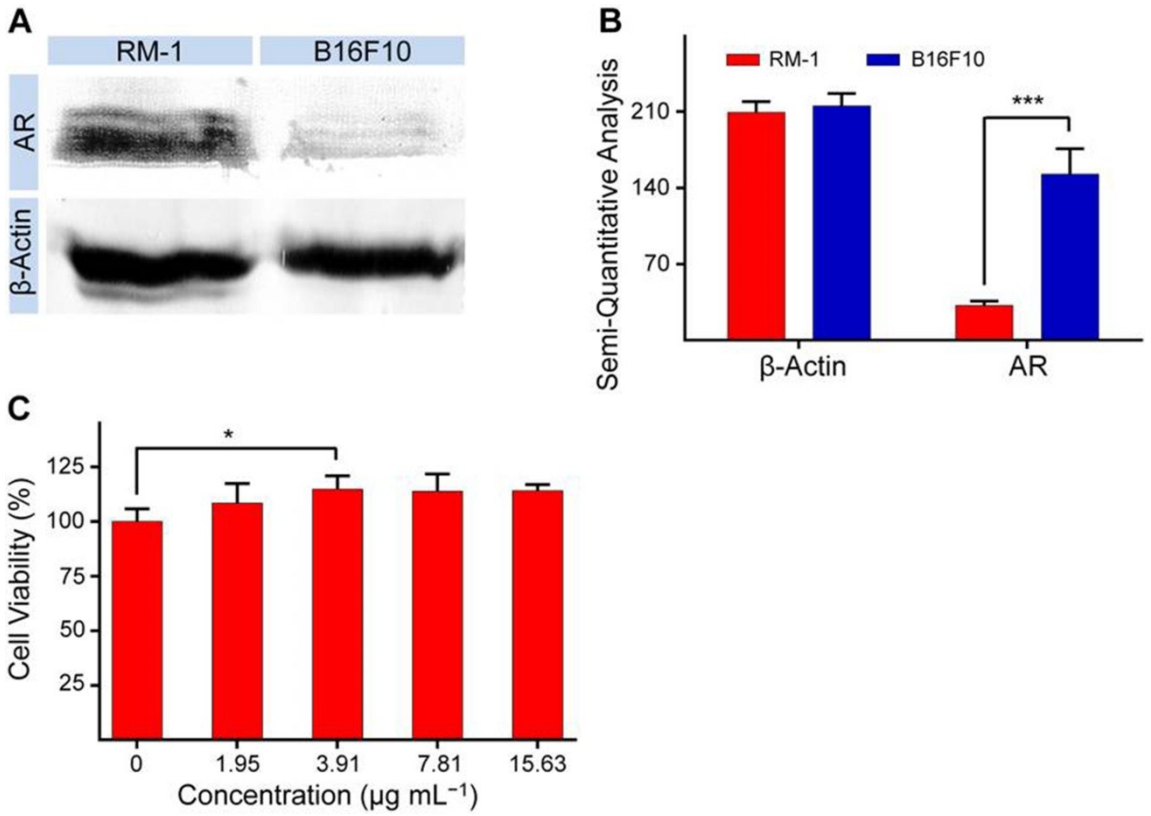


Fig. S2 AR level and TEST responsiveness of RM-1 cells. (A) Western-blot strips for AR and β-actin of B16F10 cells and RM-1 cells. AR means androgen receptor, and β-actin was used as internal reference. (B) Semi-quantitative analyses of Western blot results. (C) TEST-induced proliferation of RM-1 cells *in vitro*. Data are presented as mean ± STD (n = 6; *P < 0.05, ***P < 0.001).

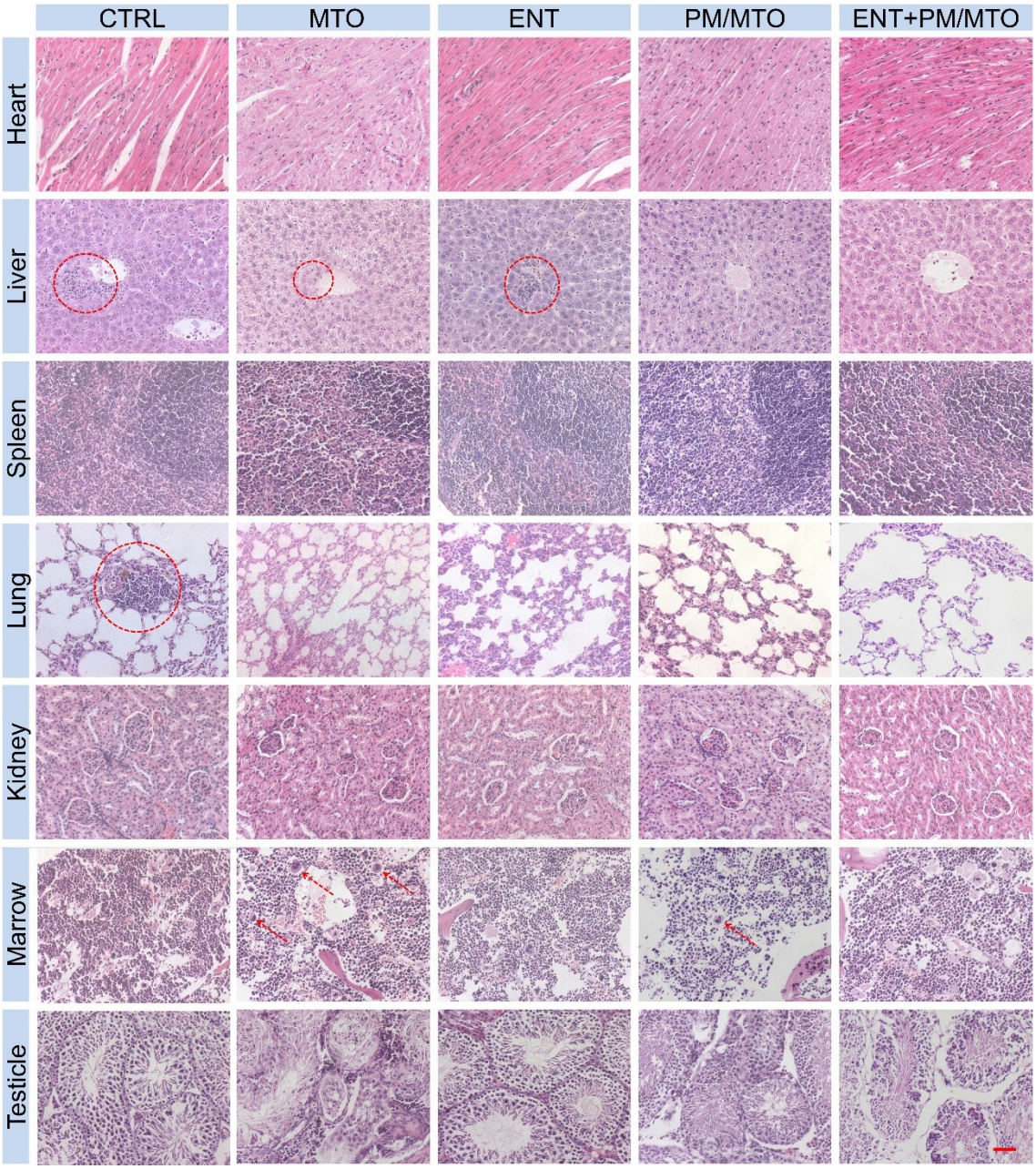


Fig. S3 Histopathology analyses of main organs. H&E staining sections of the heart, liver, spleen, lung, kidney, sternum, and testis. The red circles in the liver and lung show the metastases of PCa, and red points in marrow point out the bone marrow micronucleus. Scale bar means 100 μm.

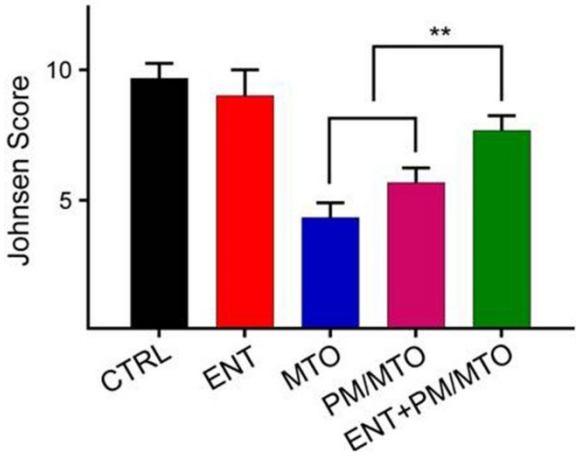


Fig. S4 Johnsen score evaluation. Johnsen scores for CTRL, ENT, MTO, PM/MTO, and ENT+PM/MTO groups. Data are presented as mean ± STD (n = 6; $^{**}P < 0.01$).