

Supporting Information

A dynamic assembly-based logic-gated tetrahedral DNzyme prodrug for mitochondrial metabolic intervention and enhanced chemodynamic therapy

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Materials. All chemicals and reagents used in this study were of analytical or molecular biology grade. All oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co., Ltd. Hemin modified DNA were synthesized by Takara. The detailed sequence information is provided in Table S1. Phosphate buffered saline (PBS) was purchased from Hyclone (USA). The polyacrylamide gel preparation kit was acquired from Solarbio Biotechnology Co., Ltd. Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), potassium chloride (KCl), tris (hydroxymethyl) aminomethane (Tris), and sodium chloride (NaCl) were obtained from Sinopharm Chemical Reagent Co., Ltd. TE buffer solution was sourced from Sangon Biotech (Shanghai) Co., Ltd. The Lactate Assay Kit-WST and Glycolysis/OXPHOS Assay Kit were from Dongren Chemical Technology (Shanghai) Co., Ltd. The extracellular oxygen consumption assay kit was supplied by Abcam plc. Rhod-2 AM, a cell-permeant calcium ion fluorescent probe, was purchased from Yeasen Biotechnology (Shanghai) Co., Ltd. Mito-Tracker Green and the mitochondrial membrane potential assay kit (JC-1) were obtained from Beyotime Biotechnology Co., Ltd. The reactive oxygen species (ROS) assay kit, MTT kit, and apoptosis kit were from Nanjing KeyGen Biotech Co., Ltd. (3-Azidopropyl) triphenylphosphonium bromide was acquired from Shanghai Yuanye Bio-Technology Co., Ltd. Fetal bovine serum was purchased from Shanghai Yudu Biological Technology Co., Ltd. Trypsin-EDTA and RPMI 1640 medium were obtained from Thermo Fisher Scientific (China) Co., Ltd.

Cell Culture. The 4T1 (breast cancer cells) and HC11 (mammary epithelial cells) used in this study were obtained from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37 °C in a humidified incubator with 5% CO_2 .

Mitochondrial Targeting Study. High-resolution fluorescence microscopy was used to evaluate the mitochondrial targeting of Cy5-labeled PETS. Briefly, 5×10^4 4T1 cells were seeded into a culture dish and incubated at 37 °C. Then, 400 μL of 200 nM

PETS was added and incubated with the cells for 12 h. After washing three times with PBS, the cells were stained with pre-warmed MitoTracker stain for 30 min,

Detection of ROS, Ca^{2+} , and Mitochondrial Membrane Potential. ROS detection. Intracellular ROS production was detected by inverted fluorescence imaging. 5×10^4 4T1 and HC11 cells were seeded into 6-well plates and cultured for 12 h. The cells were then treated with 500 nM PETS for 12 h, washed three times with PBS, stained with DCFH-DA, and imaged using an inverted fluorescence microscope. Ca^{2+} detection. 5×10^4 4T1 and HC11 cells were seeded into 6-well plates and cultured for 12 h. After treatment with 500 nM PETS for 12 h, the cells were washed three times with PBS, stained with Rhod-2 AM probe, and imaged using an inverted fluorescence microscope. Mitochondrial membrane potential detection: 5×10^4 4T1 and HC11 cells were seeded into 6-well plates and treated with 500 nM PETS for 12 h. The cells were then washed three times with PBS, stained with JC-1 probe, and detected using an inverted fluorescence microscope. All images were acquired under identical imaging settings within the same experiment.

Cell Migration Assay. 4T1 and HC11 cells were seeded into 6-well plates. When the cells reached 70%–80% confluence, a straight scratch was made in the monolayer using a pipette tip. The dislodged cells were washed away with PBS, and 500 nM PETS was added to the wells. The cells were then incubated for 0, 12, 24, and 36 h. The width of the scratch was observed and recorded under a microscope, and the images were analyzed using ImageJ software.

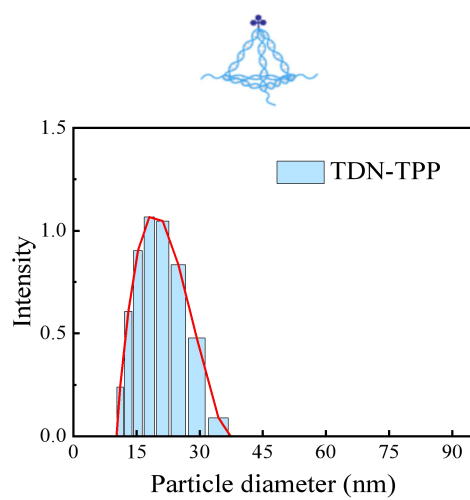


Figure S1. DLS characterization of TDN-TPP.

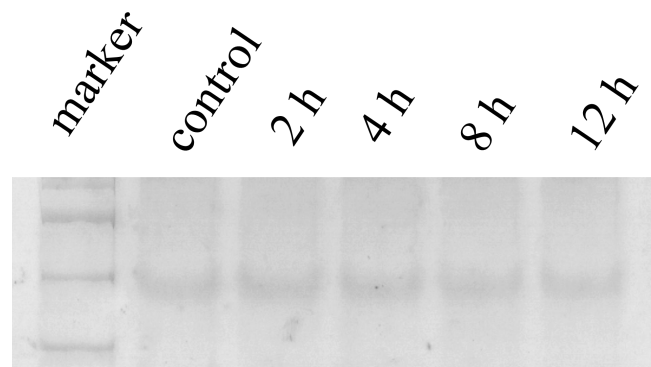


Figure S2. Stability characterization of PETS. The PETS was incubated in RPMI 1640 medium containing 10% fetal bovine serum at 37°C for different time.

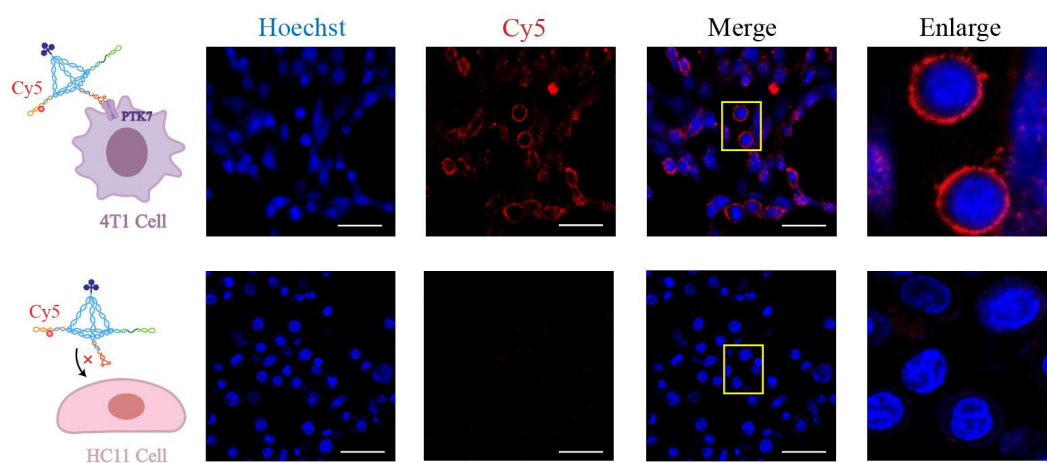


Figure S3. CLSM images of PETS for targeted binding to tumor cells.

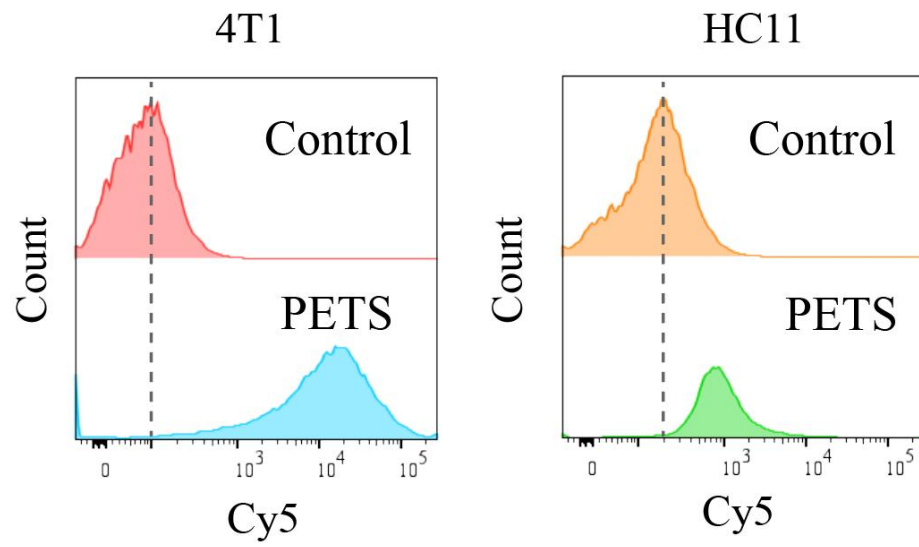


Figure S4. Flow cytometry characterization of PETS uptake by tumor cells (4T1) and normal cells (HC11).

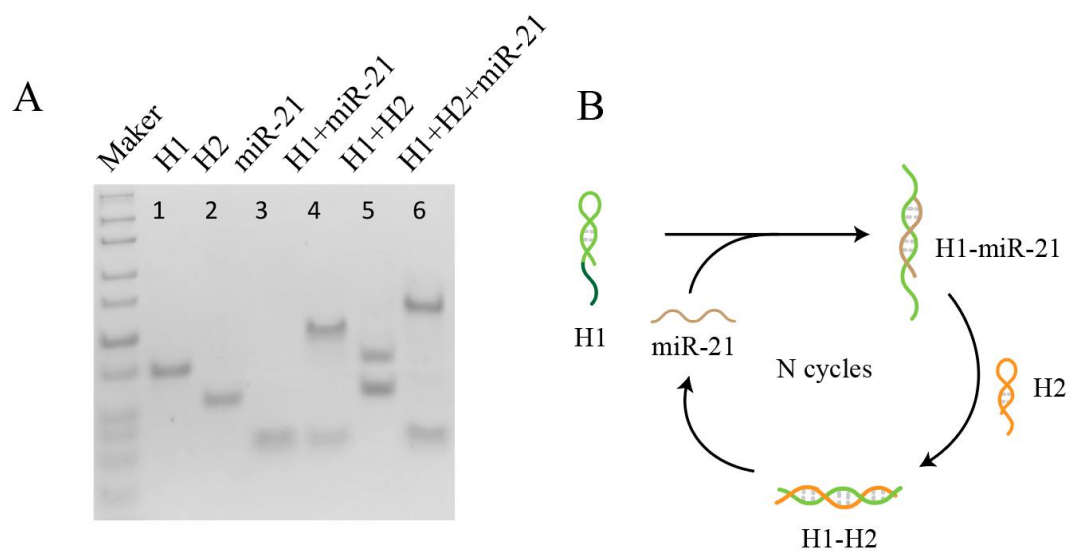


Figure S5. PAGE characterization of miR-21-triggered DNA dynamic assembly without TDN.

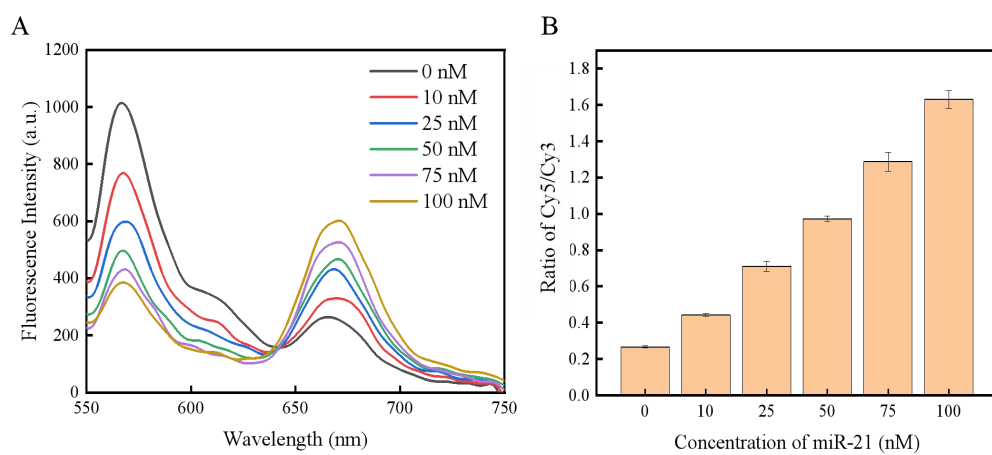


Figure S6. (A) Fluorescence spectroscopy characterization of PETS dynamic assembly at different concentrations of miR-21. (B) The fluorescence intensity ratio of Cy5 to Cy3 at different concentrations of miR-21.

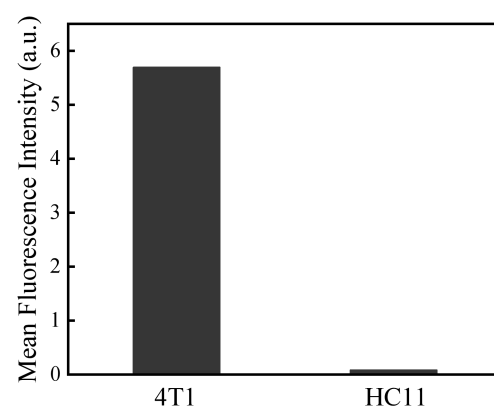


Figure S7. Fluorescence ratio of CLSM in Figure 2E.

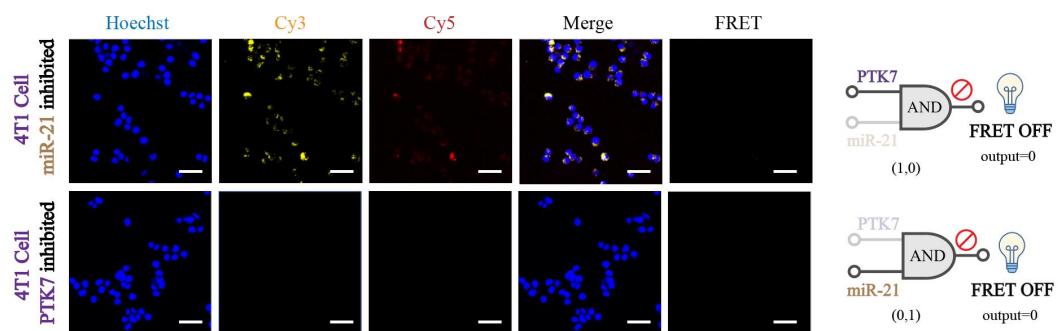


Figure S8. CLSM images of logic-gate-responsive DNA dynamic assembly in miR-21 or PTK7 inhibited 4T1 cells. The scale bar is 50 μm .

Truth Table

Input		Output
PTK7	miR-21	
0	0	0
0	1	0
1	0	0
1	1	1

Figure S9. Truth table of logic-gate-responsive DNA dynamic assembly.

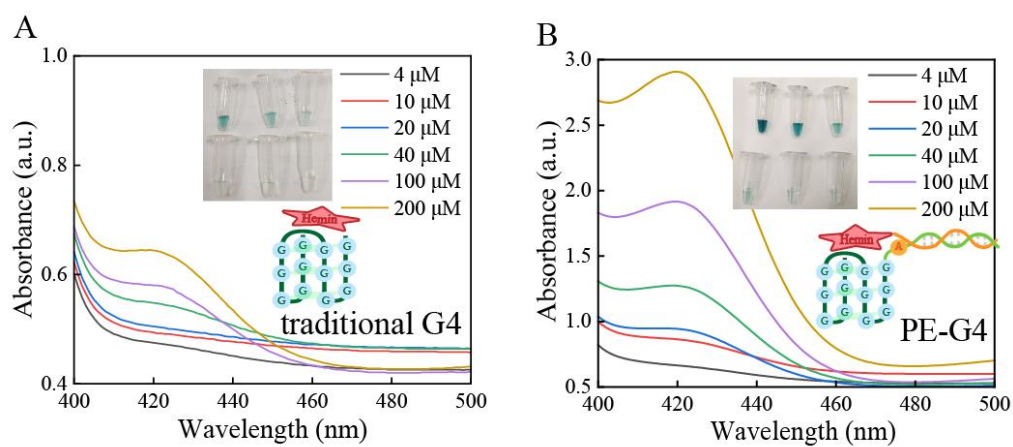


Figure S10. UV-vis spectra of ABTS oxidation catalyzed by (A) traditional G4 DNAzyme and (B) PE-G4 DNAzyme under varying H_2O_2 concentrations.

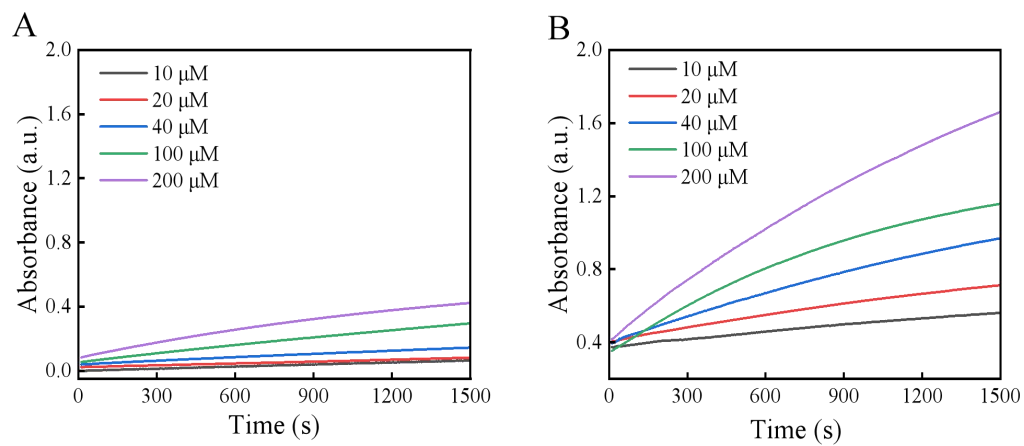


Figure S11. Time-absorbance curves at different H_2O_2 concentrations of (A) traditional G4/hemin and (B) PE-G4/hemin.

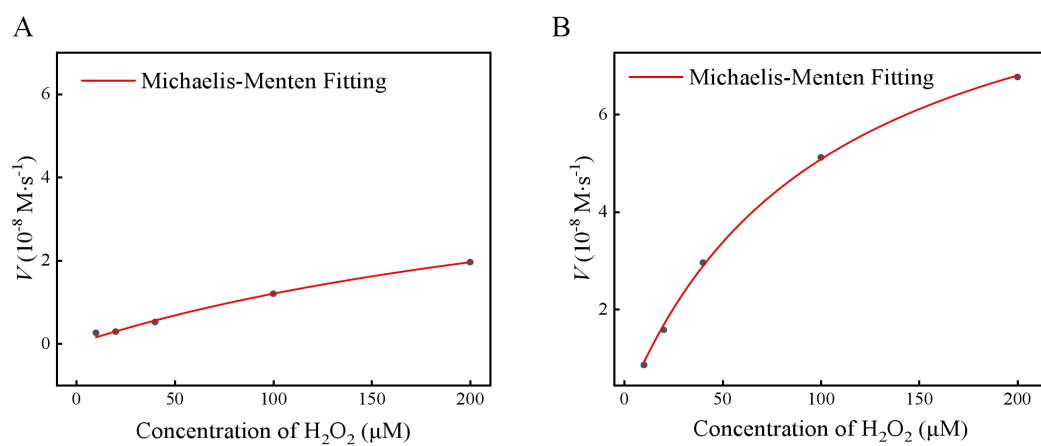


Figure S12. Michaelis-Menten curve of (A) traditional G4/hemin and (B) PE-G4/hemin.

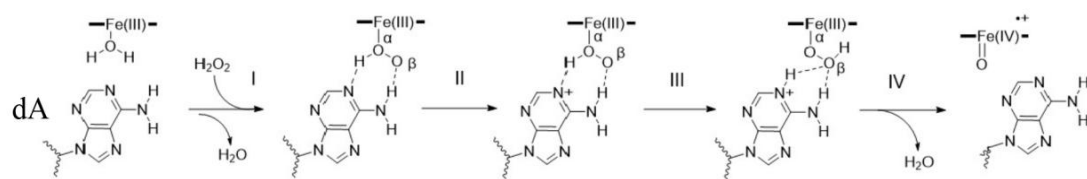


Figure S13. dA-mediated acceleration of the peroxidase catalytic mechanism of G4 DNAzyme. In G4 DNAzyme, a water molecule coordinates with the central iron of heme as a distal ligand. Initially, H_2O_2 binds to the ferric iron, displacing the water molecule while donating a proton from the α -O (bound to heme iron) to the nitrogen atom of dA (Step I). Subsequently, the adjacent 6-amino group in dA acts as a proton donor to provide a hydrogen bond to the β -O (Step II). This is followed by proton transfer from α -O to the nitrogen atom of dA, resulting in deprotonation of H_2O_2 and formation of an iron-peroxide complex (Step III). Finally, the charged dA serves as an acid catalyst to donate a proton to the oxygen atom of the iron-peroxide complex, concomitant with heterolytic cleavage of the O–O bond (Step IV).

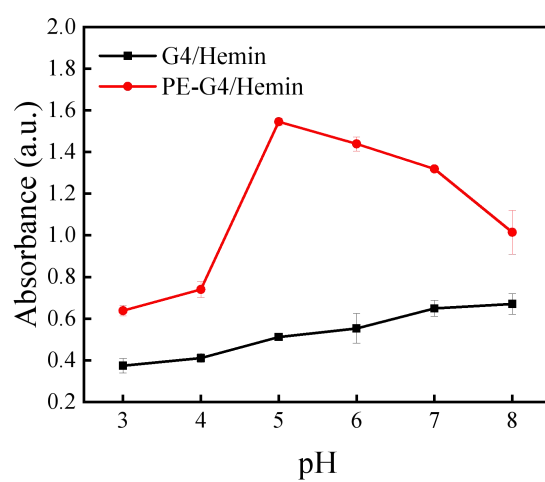


Figure S14. UV-vis spectra of ABTS oxidation catalyzed by PE-G4 and traditional G4 under varying pH conditions.

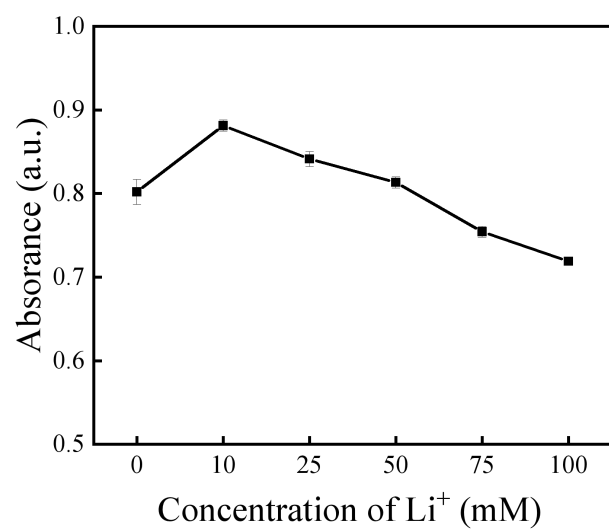


Figure S15. UV-vis spectra of ABTS oxidation catalyzed by PETS under varying Li^+ concentrations.

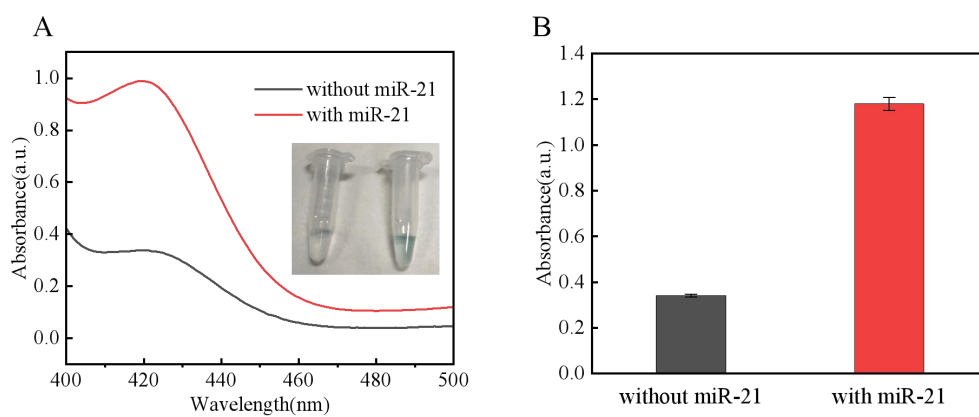


Figure S16. Feasibility characterization of Fenton-like reaction initiated by the miR-21 triggered dynamic assembly of PETS. Without miR-21, the solution was nearly colorless with an absorbance of approximately 0.3 at 420 nm, indicating that the Fenton-like reaction was not triggered. Upon addition of miR-21, a significant color change occurred, and the absorbance at 420 nm increased to 1.0, demonstrating that miR-21 induced the dynamic assembly of PETS, triggering the Fenton-like reaction.

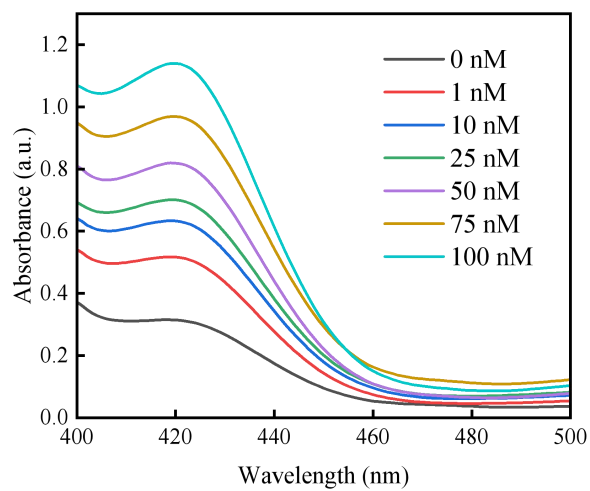


Figure S17. UV spectra of the changes of absorbance at various miR-21 concentrations.

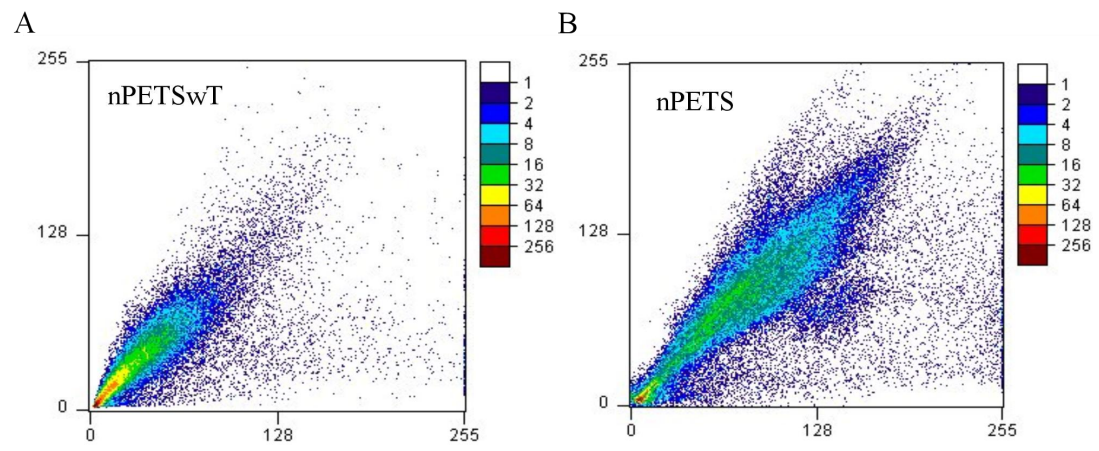


Figure S18. Colocalization scatter plots of (A) nPETSwT and (B) nPETS with mitochondria in 4T1 cells.

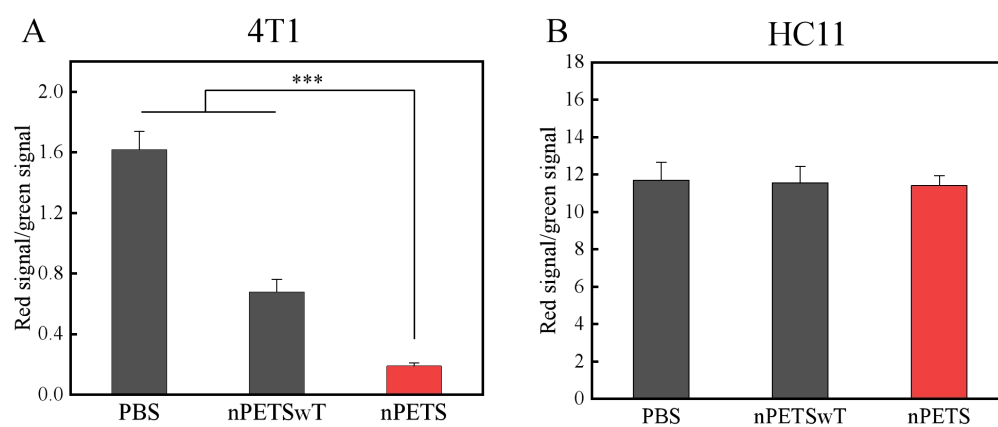


Figure S19. MMP analysis of PBS, nPETSwT and nPETS treated (A) 4T1 cells and (B) HC11 cells with JC-1 probe.

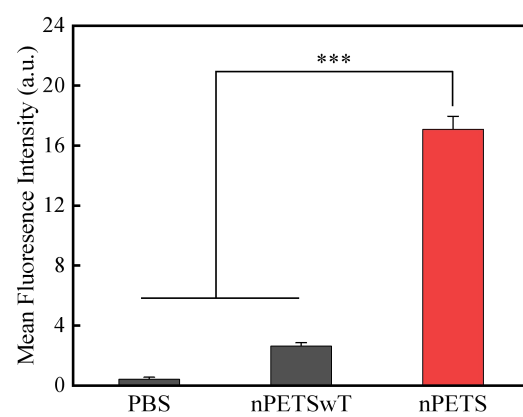


Figure S20. ROS analysis in 4T1 cells with DCFH-DA probe.

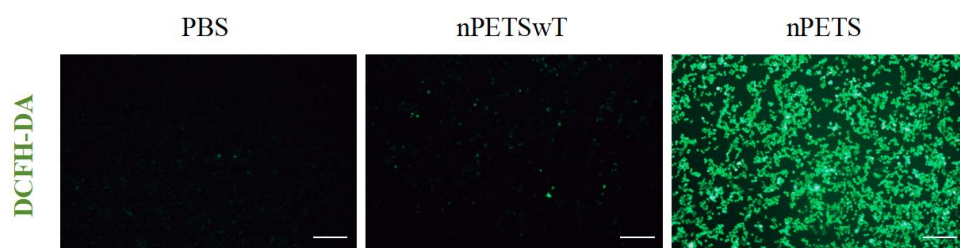


Figure S21. DCFH-DA fluorescence images under severe hypoxia (1% O₂). The scale bar is 200 μ m.

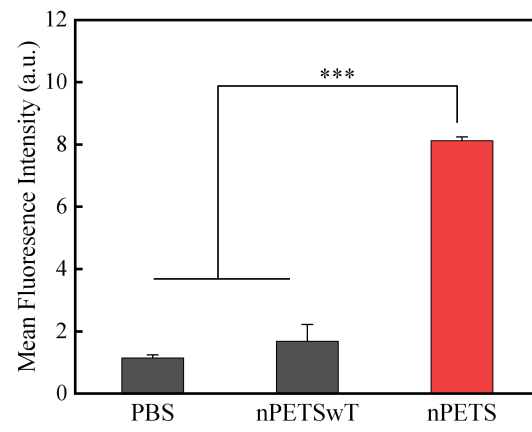


Figure S22. Ca^{2+} analysis in 4T1 cells with Rhod-2, AM probe.

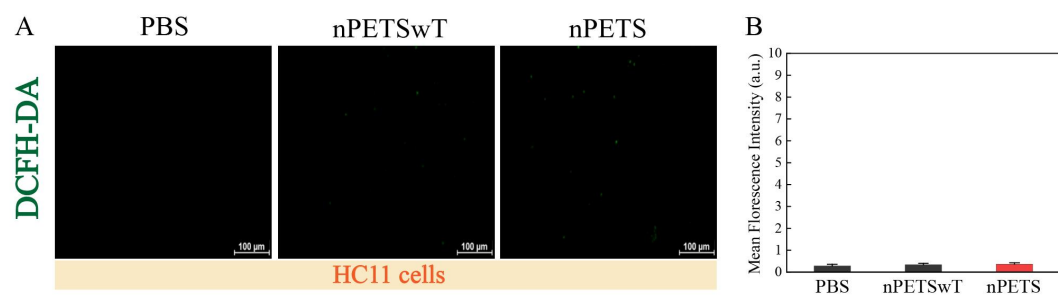


Figure S23. (A) Fluorescence microscope images and (B) of ROS generation of HC11 cells by different treatment groups.

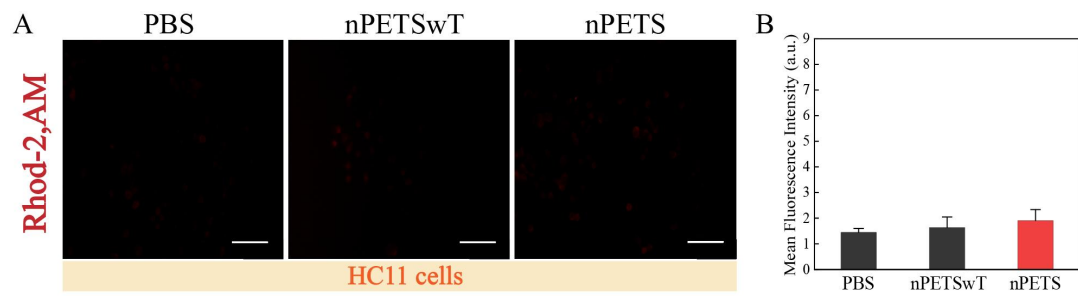


Figure S24. (A) Fluorescence microscope images and (B) analysis of Ca^{2+} generation of HC11 cells by different treatment groups.

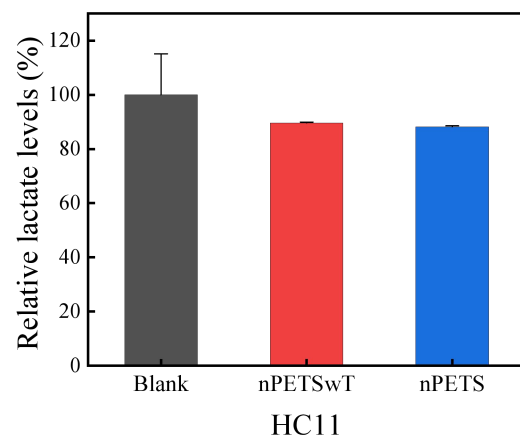


Figure S25. Analysis of lactate expression levels of HC11 cells treated with nPETSwt and nPETS.

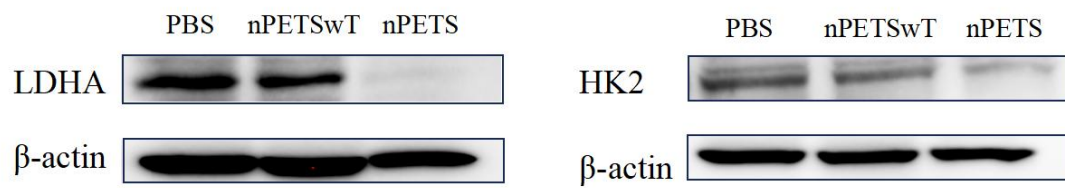


Figure S26. Western blot and quantitative analysis of HK2 and LDHA expression in 4T1 cells treated with PBS, nPETSwT, and nPETS.

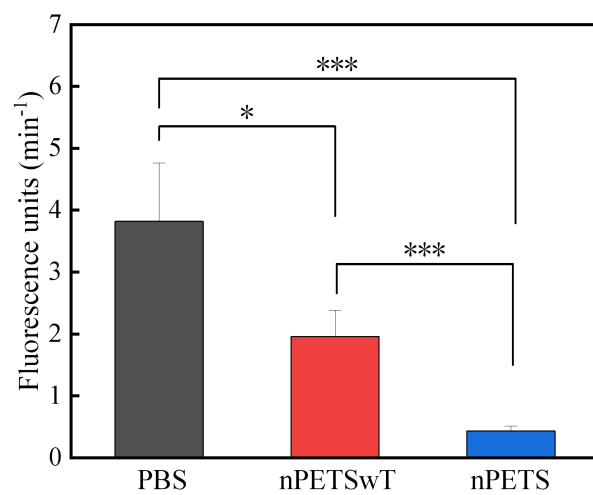


Figure S27. Detection of oxygen consumption over time in 4T1 cells treated with nPETSwt and nPETS.

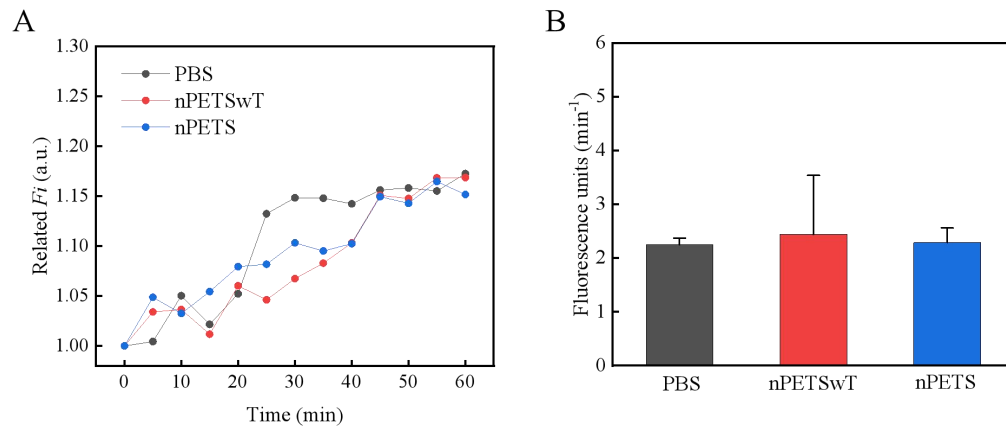


Figure S28. Detection of oxygen consumption over time in HC11 cells treated with nPETSwT and nPETS.

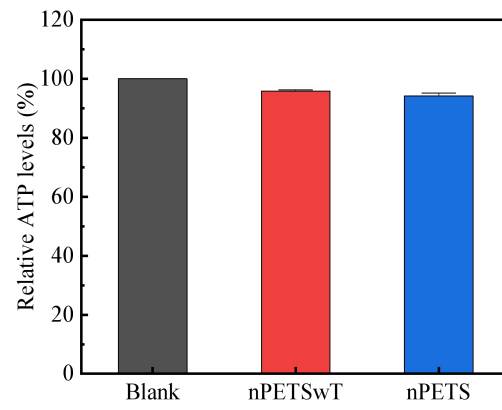


Figure S29. ATP analysis of HC11 cells treated with nPETSwt and nPETS.

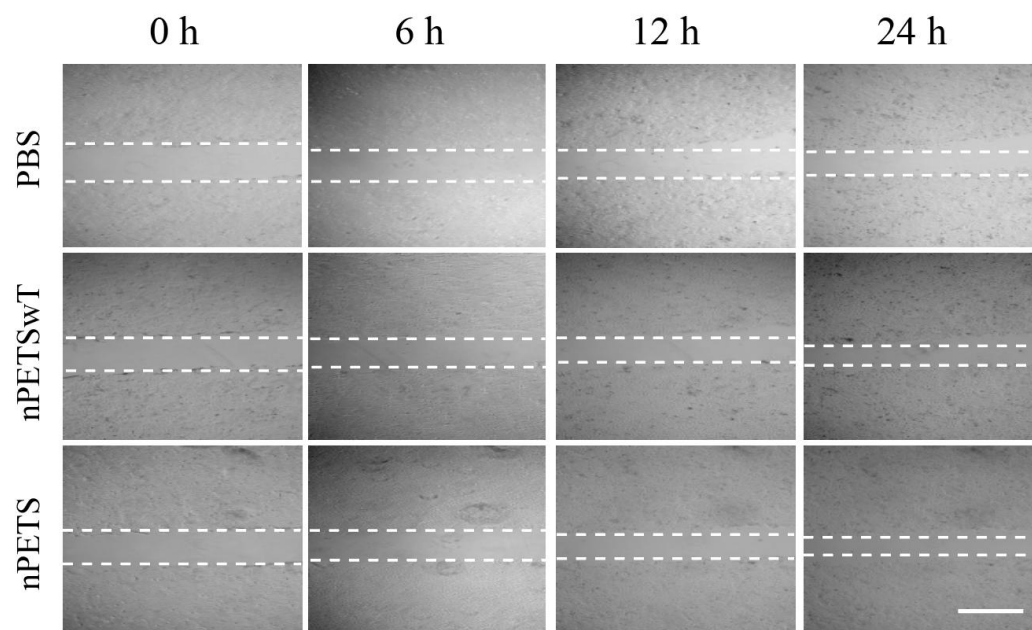


Figure S30. Cell migration analysis of HC11 cells treated with nPETSwt and nPETS.

The scale bar is 500 μm .

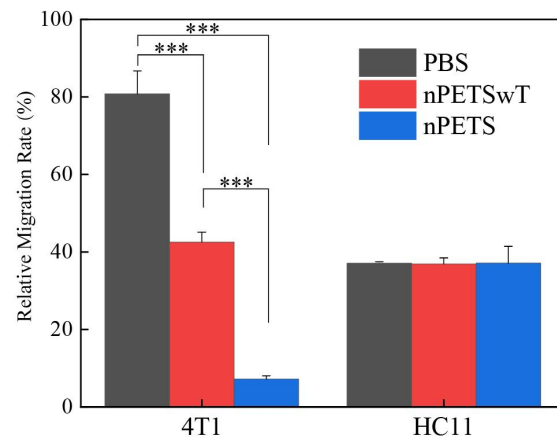


Figure S31. Quantitative analysis of cell migration.

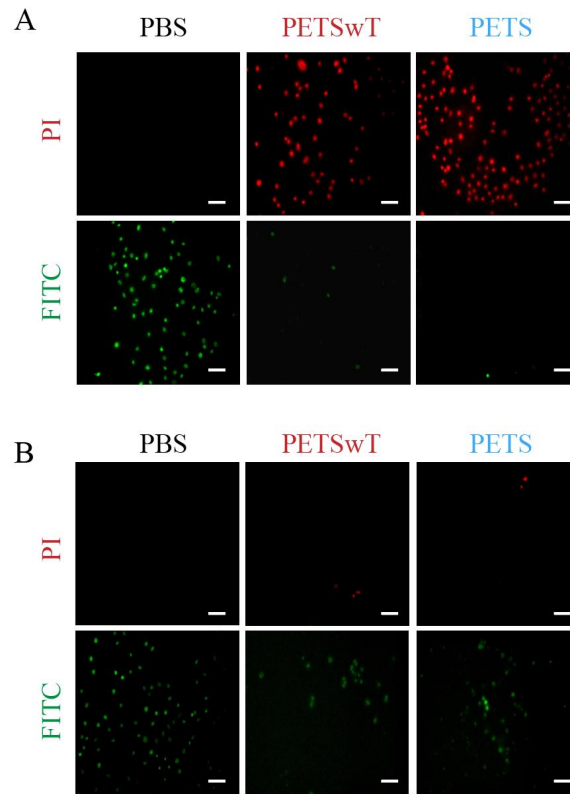


Figure S32. Apoptosis assay of (A) 4T1 cells and (B) HC11 cells treated with PETSwT and PETS. The scale bar is 100 μ m. The results showed that compared to the PBS-treated group, both PETSwT and PETS treated 4T1 cells exhibited pronounced red fluorescence (Figure S32A), indicating apoptosis induction through dynamic assembly reactions. In contrast, HC11 cells treated with either PETSwT and PETS displayed only faint red fluorescence (Figure S32B), demonstrating no significant apoptosis occurrence.

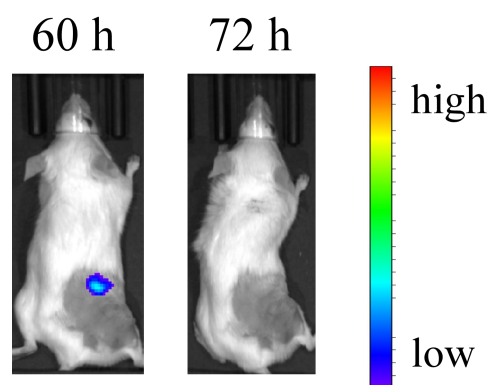


Figure S33. In vivo fluorescence images of 4T1 tumor-bearing mice at 60 and 72 h after intravenous injection of Cy7-labeled PETS.

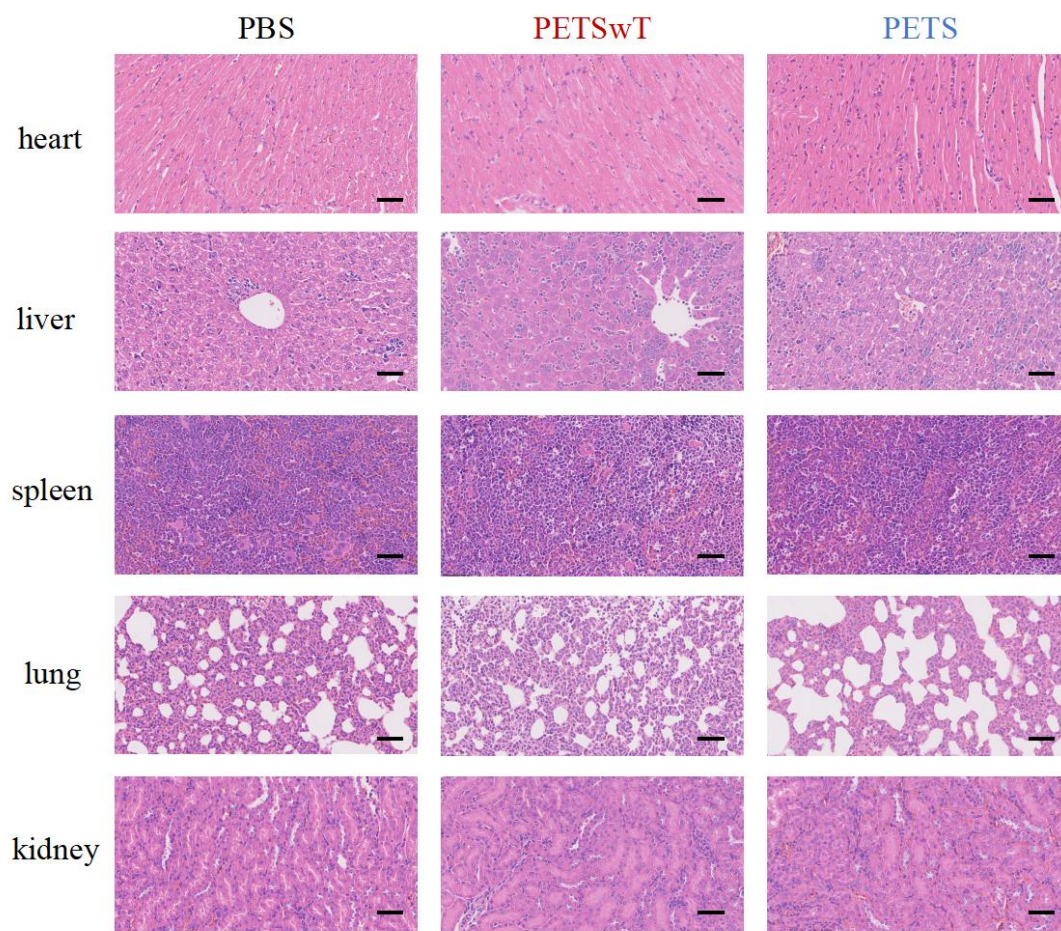


Figure S34. H&E staining plots of the main organ sections of the mouse. The scale bar is 50 μm .

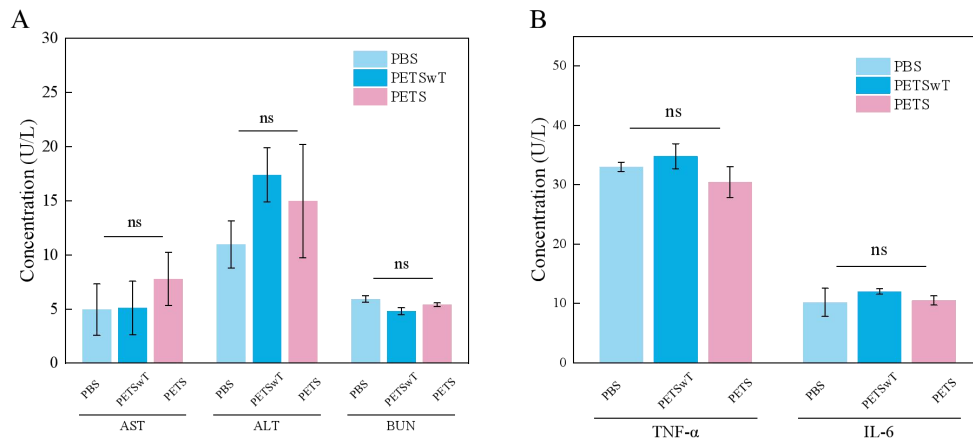


Figure S35. Evaluation of serum biochemical parameters and pro-inflammatory cytokines following different treatments. (A) Serum levels of ALT, AST, and BUN in 4T1 tumor-bearing mice treated with PBS, PETSwT, or PETS. (B) Serum levels of IL-6 and TNF- α in the different treatment groups. Data are presented as mean $\pm$ SD, $n = 3$. ns, not significant.

Table S1. Details of the DNA sequences

Oligo Name	Sequences (5'→3')
S1	TCGAACGTAGCATCATCGTTTTATCACCAGGCAGTTGACAGTGTAGCA AGCTGTAATAGATGCGAGGGTCCAATAC
S2	GTCGACTGAGTACGTCCATTTTCAACTGCCTGGTGATAAAACGACACT ACGTGGGAATCTACTATGGCGGCTCTTC
S3	TTATTCAGACTTAGGAATGTGCTTCCCACGTAGTGTGCGTTTGTATTGG ACCCTCGCAT
S4	CTCGGTCAGTGCACTGTTTTACATTCCCTAAGTCTGAAACATTACAGCT TGCTACACGAGAAGAGCCGCCATAGTA
DBCO-S3	DBCO-TTATTCAGACTTAGGAATGTGCTTCCCACGTAGTGTGCGTTTGTA TTGGACCCTCGCAT
Apt	TGGACGTACTCAGTCGACTTTTATCTAACTGCTGCGCCGCCGGGAAAA TACTGTACGGTTAGA
H1	CGATGATGCTACGTTTCGATTTTGGGAAGGGCGGGATGGGATCAACAT CAGTCTGATAAGCTAGACTGATGTTGATAGCTTATCAGACTG
H2	CAGTGCACTGACCGAGTTAATTGATAAGCTATCAACATCAGTCTAGCT TATCAGACTGATGTTGA
H2-hemin	CAGTGCACTGACCGAGTTAATTGATAAGCTATCAACATCAGTCTAGCT TATCAGACTGATGTTGA-hemin
miR-21	UAGCUUAUCAGACUGAUGUUGA

Table S2. The specific features and functions of different structural models derived from PETS

Construct	Apt/PTK7 targeting	TPP/mitochondria targeting	H1/H2 miR-21 assembly	Hemin/PETSzyme	Primary use
PETS	Yes	Yes	Yes	Yes	Full therapeutic prodrug
PETSwT	Yes	No	Yes	Yes	Control without mitochondrial targeting
PETSwA	No	Yes	Yes	Yes	Control for aptamer-mediated tumor targeting
nPETS	Yes	Yes	Yes	No	Hemin-free control for mitochondrial interference
nPETSwT	Yes	No	Yes	No	Hemin-free, TPP-free control