Supporting Information

Ferroptosis/pyroptosis Dual-inductive Combinational Anti-cancer Therapy Achieved by Transferrin Decorated NanoMOF

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Experiments and Methods

Reagents and Instruments

Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and Terephthalic acid were obtained from Aladdin-Reagent Co. Ltd. (Shanghai, China). Piperlongumine, Deferxomaine (DFO) and glutathione were bought from Bide Pharmatech Ltd. (Shanghai, China). Ferrostatin-1(Fer-1) was purchased from Macklin (Shanghai, China). Ac-DEVD-CHO (a caspase-3 inhibitor) was supplied by MedChemExpress (New Jersey, USA). z-YVAD-FMK (a caspase-1 inhibitor) was bought from Yeasen Biotech co., ltd (Shanghai, China). MTT reagent and DAPI were purchased from Sigma-Aldrich Inc. (St Louis, MO). β-actin antibody, anti-mouse IgG (H +L), anti-rabbit IgG (H+L), ROS detection kit (DCFH-DA), Membrane Protein Extraction Kit, Coomassie Blue, BCA protein assay kit, GSH and GSSG Assay Kit, LDH Assay Kit were provided by Beyotime Institute of Biotechnology (Jiangsu, China). Intracellular Iron Colorimetric Assay Kit was obtained from Applygen Inc. (Beijing, China). NP40 lysis buffer and cocktail protease inhibitor were acquired from Merck (Darmstadt, Germany). GPX4 antibody was bought from Signalway Antibody (MD, USA). HMGB1 antibody was supplied by Absin Bioscience Inc. (Shanghai, China). GSDMD antibody was provided by Abcam. BODIPY581/591-C11 was purchased from Thermo Fisher (Waltham, MA). RPMI 1640 medium (RPMI), fetal bovine serum (FBS), penicillin/streptomycin (100 U/mL), phosphate buffered solution (PBS) and Tyrisin 0.25%-EDTA were obtained from Biosun Biotechnology Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade and used without further purification.

Cells and Animals

4T1 cells (mouse breast cancer) was obtained from the Institute of Biochemistry and Cell Biology (IBCB, Shanghai, China). Balb/c mice were purchased from Shanghai Lingchang Laboratory Animal Co. Ltd. (Shanghai, China).

Animal Care

The procedures for care and use of all animals were approved by the Ethics Committee of the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University and all applicable institutional and governmental regulations concerning the ethical use of animals were followed**.**

Preparation of MOF(Fe)

To fabricate nanosized metal-organic framework, we applied a novel and straightforward method to fabricate the MOF (Fe) as reported[1]. In detail, 10 mL $FeCl_3 \cdot 6H_2O$ solution (H_2O, 40 mM) and 10 mL TPA solution (DMF,40 mM) were delivered into 25 mL initial solution (DMF/water=1) separately by peristaltic pump in 15 min under 50 °C water bath in the three-necked bottle. Then, 30 mL of reaction solution was taken out from the reaction bottle followed by adding 10 mL of $FeCl_3 \cdot 6H2O$ stock solution and 10 mL TPA stock solution again in 15 min. After that, 25 mL of reacting product was taken out, and former step was repeated for the last time. The final product was collected by centrifuging under 12000 rpm and washed by DMF as well as ethanol respectively. Then the collected MOF was dissolved in double

distilled water to make the 1mg/mL preparation.

Lipid layer and LipoMof Preparation

DOPE, OA and Cholesterol were dissolved and mixed in chloroform in molar ratio of 2:1:1, respectively. The mixture was evaporated under reduced pressure at 40℃.Then the residue was hydrated by double distilled water and sonicated for 15s. MOF stock solution was then incubated with liposome solution for 2 hours (v/v=1/1). The mixture was centrifuged under 500 rpm and washed by double distilled water twice to get LipoMof

Synthesis of Tf-LipoMof@PL nanoparticles

Since piperlongumine is hard to dissolve in distilled water, itwas first dissolved in anhydrous ethanol to make 3 mg/mL stock solution. Then the needed amount of piperlongumine stock solution was incubated with MOF stock solution to prepare MOF@PL of designed concentration (calculated by piperlongumine) overnight. pH-sensitive liposome solution was then added into MOF@PL (w/w=1/1) solution followed by incubating for 2 hours under room temperature and centrifuge under 500 rpm to get LipoMof@PL. Then transferrin was put into LipoMof@PL solution and incubate for 2 hours under room temperature, extra transferrin was removed by centrifuging under 1000rpm for 5 min. Then the loading capacity of transferrin was tested by BCA analysis. Transmission electron microscope (FEI, Hillsboro, Oregon, USA) was acquired for morphology and nanoparticles size. To study the stability of the

nanoparticle, nanoparticles were stored at 4 °C for 14 days and the diameter was recorded.

Fluorescence Release Experiment

To validate the successful coating and testify the sealing effects of the lipid layer, coumarin loaded MOF was coated with lipid layer and fluorescence release was measured in PBS (pH 7.4). 1 ml of coumarin loaded LipoMof was added into dialysis bag (MD1000) and put into 10 mL PBS(pH 7.4) under constant stirring at 37℃ , in which system only dye molecule can pass the dialysis membrane. The amount of released dye can be monitored by the fluorescence intensity with spectrofluorometer (Spectra MAX M3, Molecular Devices Corporation, Sunnyvale, CA, USA. Excited 377 nm, emission 470 nm). The measurement was supposed to run for 1h, and data was taken every 5 minutes. 20 μL absolute Triton-X-100 was added at 20 min into the dialysis bag. Then the lysis of lipid layer would allow coumarin to diffuse into the medium from the pore of MOF, leading to the change of fluorescence intensity.

Extracellular ·OH detection

PL, Mof@PL, LipoMof@PL and Tf-LipoMof@PL (0.5 mg/mL calculated by PL, 200 μL) was incubated with H_2O_2 (10 mM) and methylene blue (MB) (32 μg/mL) for 1h at 25 °C. MB + H_2O_2 (10 mM) was used as control. All samples were centrifuged to remove MOFs and the absorbance change of MB at 664 nm was recorded by UV−vis spectroscopy (SP-756P, Shanghai spectral instrument co. LTD).

Cell Culture

4T1 murine breast cancer cells were incubated in RPMI 1640 medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000U mL-1) at 37℃ in a humidified atmosphere containing 5% CO2.

Live/Dead Staining Experiment

4T1 cells were seeded in 6-well plates ($5*10^5$ cells per well) and incubated for 12 hours. After being treated with different formulations for 6 hours, cells were washed with PBS and incubated with PI (20 μL per well) and Hochest33324 (10 μL per well) contained medium for 10 min. Then observe under inverted microscope (Olympus, Shinjuku, Japan).

Cellular GSH Assay

4T1 cells were seeded in 6-well plates (10^6 cells per well) and incubated 12 hours. After being treated with different formulations for 6 hours, cells were washed with PBS three times and collected. Next, the cells were lysed with 200 μL cell lysis buffer per well. Then the harvested cell lysate was treated under the guidance of GSH/GSSG Assay Kit to test the amount of GSH. The percentage content of GSH was calculated based on the GSH level of control group.

***In vitro* cell uptake assay.**

4T1 cells were seeded in 6-well plate and incubated for 24 hours. Then FITC and FITC loaded Tf-LipoMof was added to treat 4T1 cells for 12 h. Intracellular fluorescence was

observed and measured by CLSM (Leica, Wetzlar, Hesse, Germany). Mean intracellular fluorescence intensity was obtained by "LAS AF Lite" software.

***In vitro* Cytotoxicity Evaluation toward Cancer Cells.**

5000 cells per well were seeded in 96-well plate with 200 μL RPMI 1640. Before further treatment, the cells were incubated at 37°C/5% CO_2 overnight for fully attachment. Then the free drug and nanoparticle groups were added. Each group (piperlongumine, Tf-LipoMof,LipoMof@PL, Tf-LipoMof@PL) in different concentration ladder (1,2,5,10,15,20 μM at equivalent dosage of piperlongumine) was tested for 6,12,24 hours, respectively. After that, standard MTT assay was used to quantitatively analyze the *in vitro* cytotoxicity. Briefly, 20 μL MTT (5mg/ml in PBS) were added into each well. 4 h's incubation later, the medium was removed and replaced by DMSO. The absorbance was obtained under 570 nm by microplate reader. The relative cell viability was calculated by formula: Cell Viability (%) = (OD_{570nm} sample/OD_{570nm} control) x 100%.

Determination of the Intracellular Iron Level

10^6 4T1 cells per well were seeded into 6-well plates and were incubated for 24 h post seeding with piperlongumine, LipoMof@PL and Tf-LipoMof@PL (10 mM per well calculated by piperlongumine) respectively. Medium was removed and the cells were washed with DPBS twice. Then the cells were collected and digested by nitric acid overnight. The intracellular iron level was tested by ICP-MS (Thermo Fisher ,Waltham,

MA).

Detection of *in vitro* ROS.

The production of intracellular ROS was determined by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) kit. 4T1 cells were seeded into a 6-well plate for 24 h. Then cells were treated with piperlongumine, LipoMof@PL and Tf-LipoMof@PL with equivalent amount of PL (10 mM) for 6 hours. Then cells were incubated with DCFH-DA for 20 min as Assay Kit suggested. Fluorescence intensity was evaluated by CLSM. Mean intracellular fluorescence intensity was obtained by "LAS AF Lite" software.

Detection of *in vitro* H_2O_2

The intracellular H_2O_2 level was detected by H_2O_2 Assay Kit through Titanium sulfate microplate method. 10^6 4T1 cells per well were seeded into 6-well plates. After incubating for 24 hours, piperlongumine, LipoMof@PL and Tf-LipoMof@PL (10 mM per well calculated by piperlongumine) were added in each well respectively (n=3). Then the intracellular H_2O_2 level was tested according to the manufacturer's guidance of the assay kit.

LPO Detection

10^6 4T1 cells per well were seeded into 6-well plates. After 24 h's incubation, piperlongumine, LipoMof@PL and Tf-LipoMof@PL were added into the medium. After incubating for 6 hours, cells were stained with BODIPY-C11 fluorescence probe

(5 μM) for 20 min, Then cells were washed with PBS three times and subjected to CLSM observation.

Western Blot Analysis

10^6 4T1 cells per well were seeded into 6-well plates. After incubating for 24 hours, piperlongumine, LipoMof@PL and Tf-LipoMof@PL (10 mM per well calculated by piperlongumine) were added in each well respectively (n=3). After 6 h's incubation, 4T1 cells were treated under standard procedure to get cell lysate. The protein amount was normalized via BCA protein concentration determination method. Then the obtained cell lysates with interested proteins (50 μg) were subjected to western blot. GPX4, GSDMD and intracellular HMGB1 level was detected by western blot and semi-quantification was done by "Image J" software.

In vitro **Cell Viability in the Presence of Different Inhibitors or Promotors of Ferroptosis and Pyroptosis**

4T1 cells were seeded inn 96-well plates with density at 5000 cells per well. After 24 h's incubation, piperlongumine, LipoMof@PL and Tf-LipoMof@PL (at an equivalent amount of 10 mM piperlongumine) were added into each well. Then 1 μM ferrostatin-1 (a ferroptosis inhibitor), 50 μM Ac-DEVD-CHO (a caspase-3 inhibitor) ,50 mM z-YVAD-FMK (a caspase-1 inhibitor), 100 μM deferoxamine (an iron chaletor), 2 mM glutamic acid (a ferroptosis promoter), 2 mM cystine (the precursor of cysteine) and 2 mM glutathione (a cofactor of GPX4) and 20 μM α-tocopherol (a LPO scavenger)

were added into each well respectively. After incubating for 12 hours, cell cytotoxicity was evaluated by MTT method.

Detection of *In vivo* Antitumor Effects

4T1 tumor bearing Balb/c mice model was established by subcutaneously injecting 4T1 cells ($1x10^7$ suspended in 100 μL DPBS) into the flank of each mouse (5-6 weeks). Mice were randomly divided into 4 groups and was intravenously given saline, piperlongumine, LipoMof@PL and Tf-LipoMof@PL (0.5 mg/kg calculated by piperlongumine), respectively after the tumor volume reached 100-150mm^3. Tumor volume (length x width x height/2) and mice body weight were measured every other day. After 22 days, mice were sacrificed. Then major organs were taken and embedded in paraffin for immunofluorescence analysis as well as H&E staining. Tumor tissue was grinded, lysed by RIPA (containing 1mM PMSF) for 30 min on ice and then filtered by 40 mm filter, and the samples were used for western blot analysis of GPX4, HMGB1 and GSDMD expression in tumor tissue.

Biodistribution

To verify the homotypic targeting of Tf-LipoMof@PL, biodistribution was studied with orthotopic 4T1 tumor models. When the tumor volume reached 250-300 mm^3, mice were intravenously injected with IR820 (500μg/kg), IR820-labeled LipoMof@IR820 and Tf-LipoMof@IR820, respectively. The mice were placed in an IVIS imaging system (PerkinElmer, Fremont, USA) for observation at the predetermined time points. At 48 h after injection, the mice were sacrificed and the major organs and tumor tissues were removed for ex vivo imaging and analysis.

Statistical Analysis

All data were presented as mean ± SD. One-way and two-way analysis of variance (ANOVA) was used to determine the statistical significance which was defined as * P < 0.05, ** P < 0.01, ***P < 0.001.

Figures

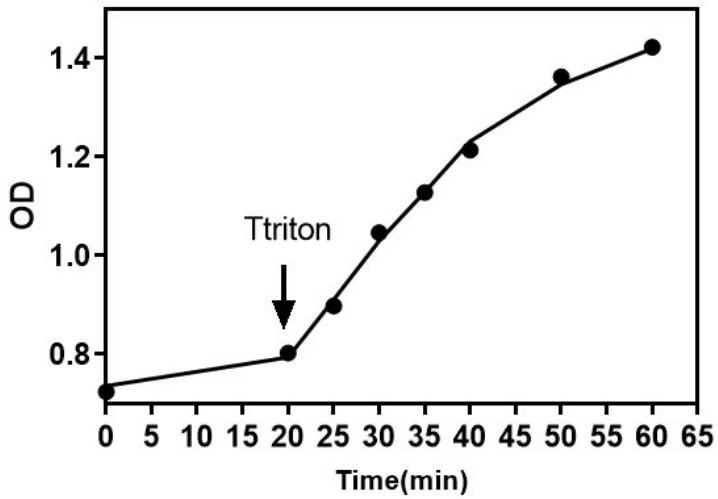

Figure S1. Fluorescein release from lipid-coated MOF nanoparticles before and after addition of Triton X-100. (n=3)

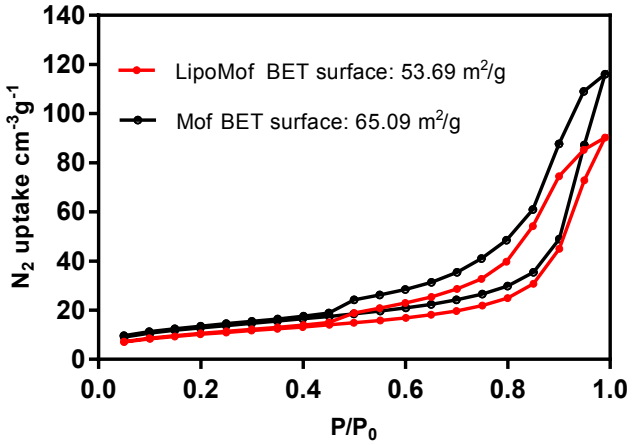

Figure S2. N2 adsorption-desorption isotherms of Mof and LipoMof.

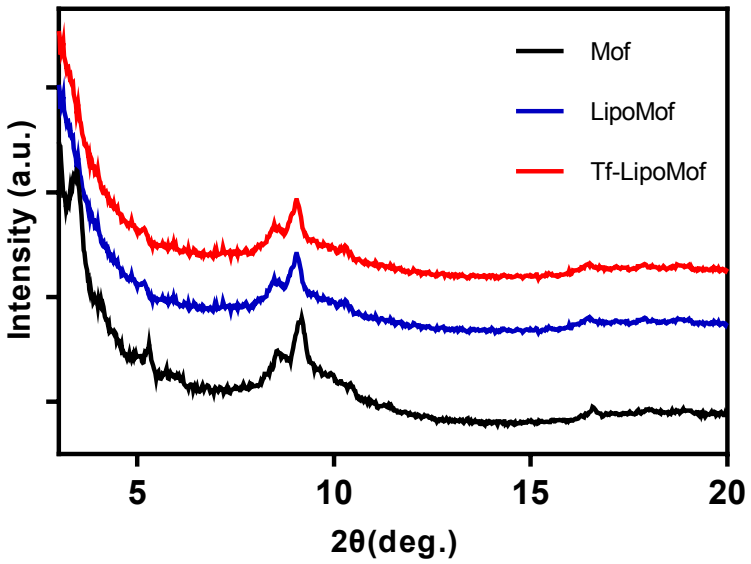

Figure S3. X-ray powder diffraction patterns of Mof, LipoMof and Tf-LipoMof.

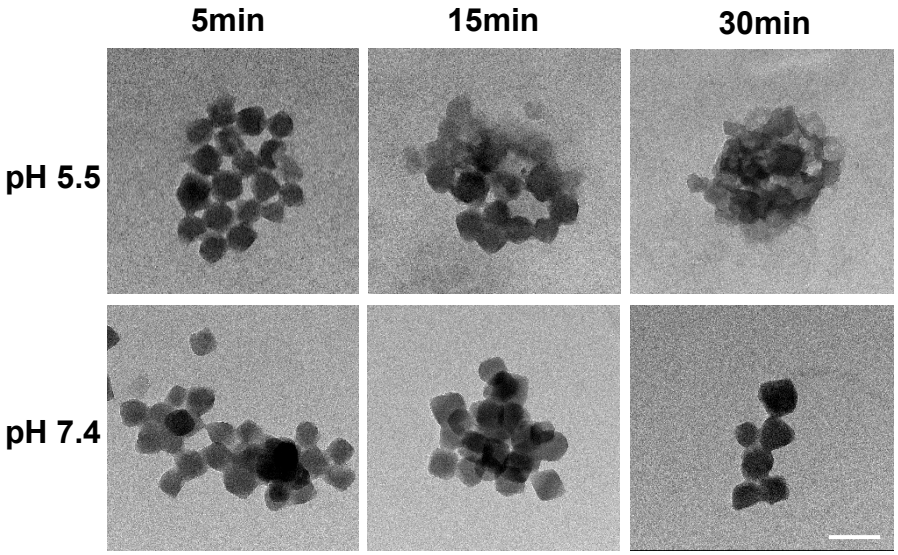

Figure S4. TEM images of LipoMof@PL under pH 5.5 and pH 7.4 at different time point.

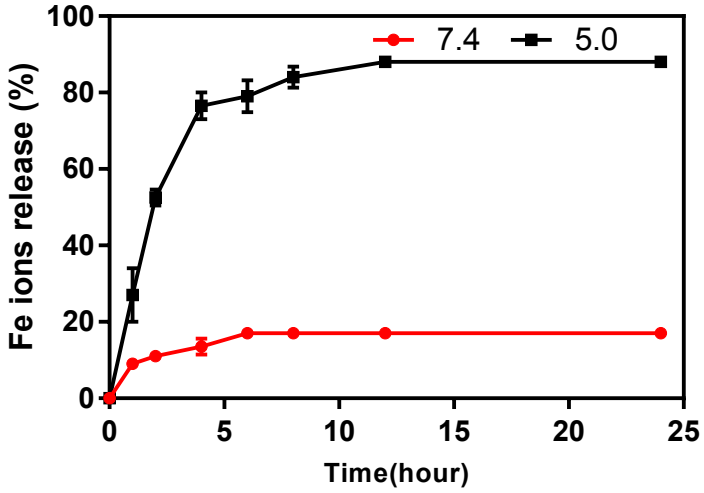

Figure S5. The release profile of Fe ions in Tf-LipoMof@PL under pH 5.5 and 7.4. (n=3)

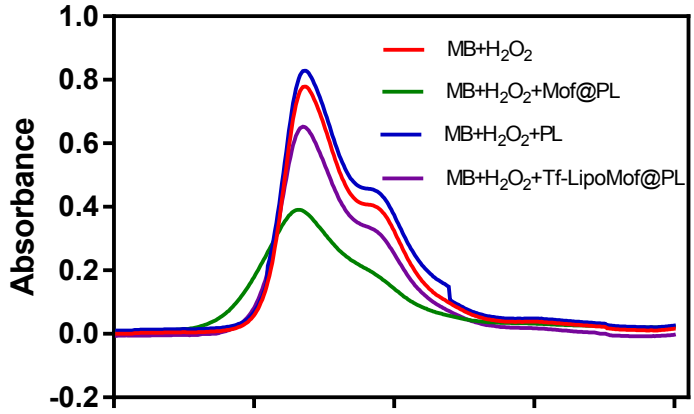

Figure S6. In vitro analysis of •OH.

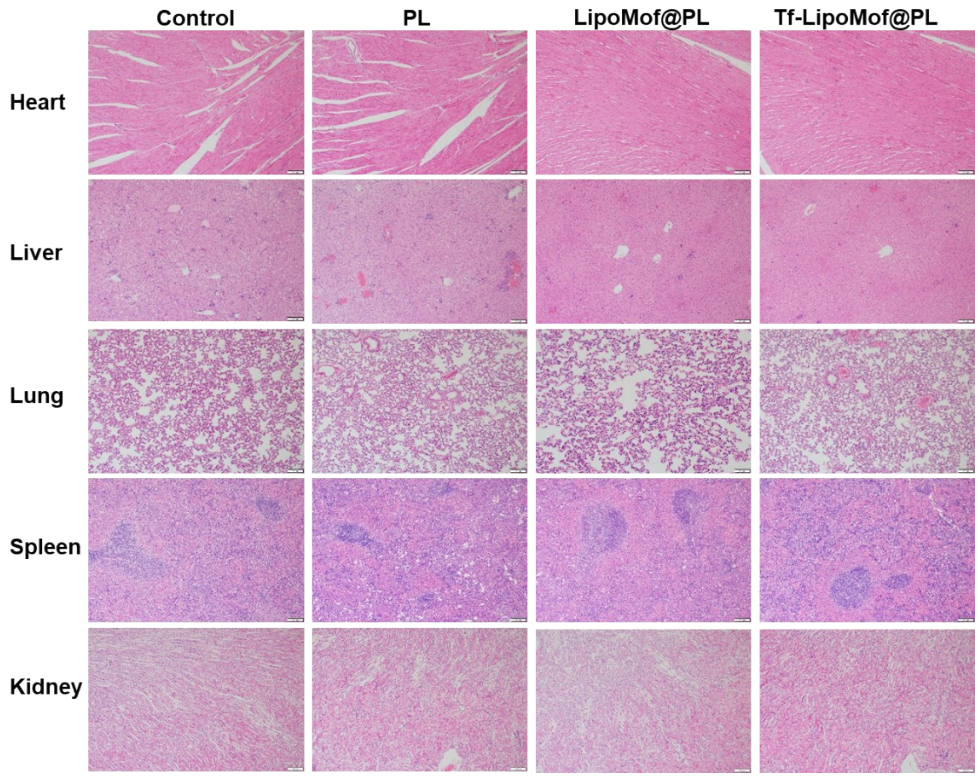

Figure S7. H&E staining of different organs obtained from BALB/C mice after various treatments. (Scale bar: 100 μm).

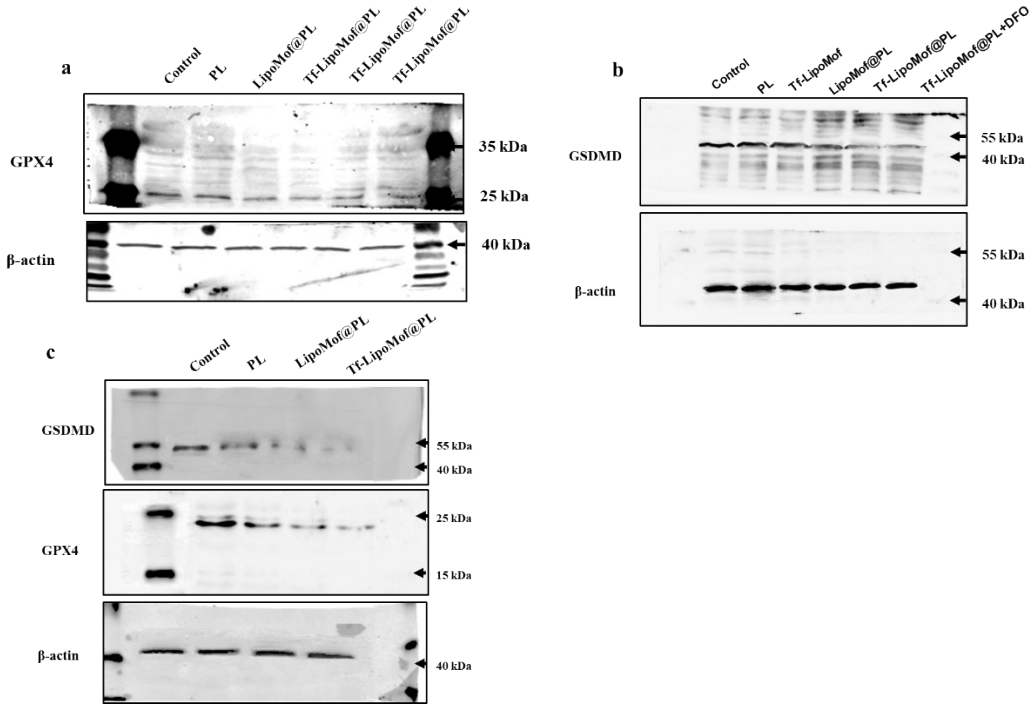

Figure S8. Full-length Western Blots. (a) Uncropped images for Figure 3d. (b) Uncropped images for Figure 3k. (c) Uncropped images for Figure 4h.

1. Wang, X. G.; Cheng, Q.; Yu, Y.; Zhang, X. Z., Controlled Nucleation and Controlled Growth for Size Predicable Synthesis of Nanoscale Metal-Organic Frameworks (MOFs): A General and Scalable Approach. *Angew Chem Int Ed Engl* **2018,** *57* (26), 7836-7840.