A Cationic Metal-Organic Framework to Scavenge Cell-Free DNA for Severe Sepsis Management

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Materials and Methods

Chemicals and bioreagents

2-methylimidazole, $Zn(NO_3)_2 \cdot 6H_2O$, PEI (600, 1800, and 25K Da), methanol, and calf thymus DNA were purchased from Sigma Aldrich. Cy7-NHS ester was purchased from Meilun (China). Phosphate buffered solution (PBS, pH 7.4), Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco (USA). HEK-Blue hTLR3, hTLR4, and hTLR9 reporter cell lines, QUANTI-Blue detection kit, CpG Bw006, Poly(I:C), and LPS were purchased from InvivoGen. TRIzol reagent, Lysotracker Red DND-99, DAPI, Quant-iT PicoGreen dsDNA Assay Kit, TNF-α, and IL-6 ELISA Kit were purchased from Invitrogen. Anti-CD11c-PE, anti-F4/80-PE-Cy7, and anti-CD11b-FITC antibodies were purchased from eBioscience. DNA Blood Mini Kits were purchased from QIAGEN (Germany). Primers for glyceradehyde-3-phosphate dehydrogenase (GAPDH) and TNF-α were synthesized by Sangon Biotech (China). Total mRNA extraction kits, TIANScript Ⅱ RT Kits, and SuperReal PreMix Plus (SYBR Green) were purchased from Tiangen (China). ROS Kits were purchased from Beyotime (China). CCK-8 was purchased from Abcam.

Instruments

Zeta potential and size of nanoparticles (NPs) were characterized with a Malvern Zetasizer. ^1H NMR spectra were recorded on an AV-300 NMR spectrometer (Bruker; Karlsruhe, Germany) in deuterated trifluoroacetic acid-d. UV-vis absorption spectra were obtained using a UV-1700 spectrophotometer (Shimadzu; Japan). Fourier transform infrared spectra (FTIR) were obtained with a Nicolet 520 FTIR spectrometer. Powder X-ray diffraction spectra were obtained with a D8 ADVANCE X-ray diffractometer (40 kV, 40 mA). Zn content was detected by inductively-coupled plasma atomic emission spectroscopy (ICP-MS, Thermo Scientific XSERIES 2, Thermo Fisher Scientific, USA).

Preparation of PEI-*g*-ZIF NPs

A solution of Zn $(NO_3)_2 \cdot 6H_2O$ (0.73 g) in methanol (50 mL) was rapidly poured into a blended solution of 2-methylimidazole (1.62 g) and PEI with different molecular weight (0.3 g) in methanol (50 mL) and stirred at room temperature for 1 h. After centrifuging at 8,000 rpm for 5 min, the precipitation was washed with methanol three times then dissolved in water for lyophilization.

DNA binding assay

Calf thymus DNA (25 μL, 5 mg/mL) and Quant-iT PicoGreen (12.5 μL) were mixed with 10 mL of TE buffer and shaken for 10 min in the dark to form a fluorescent complex. Then, 100 μL of the DNA-Quant-iT PicoGreen solution and 100 μL of materials at different concentrations were added into the wells of a 96-well plate. After incubation at 37 °C for 30 min, the fluorescence intensity of each well at 520 nm was measured with a plate reader (Bio-Tek, Winooski, VT) with excitation at 490 nm.

TLR activation assays *in vitro*

HEK-Blue hTLR3, hTLR4, and hTLR9 reporter cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For inhibition of TLR9 activation by materials, HEK-Blue hTLR9 cells in DMEM with or without FBS were plated at a density of 8×10^4 cells/well in a 96-well plate for 30 min, then 2 μL of CpG BW006 at 1 mg/mL was added. After 20 min of incubation, 2 μL of materials at different concentrations was added in a final volume of 200 μL. At 24 h, supernatants were collected and incubated with QUANTI-Blue. The embryonic alkaline phosphatase (SEAP) activity in each well was determined by measuring the OD at 620 nm using a plate reader.

For materials inhibition of hTLR3 activation, HEK-Blue hTLR3 reporter cells in DMEM with or without FBS were plated at a density of 5×10^4/well in a 96-well plate for 30 min, then 2 μL of poly(I:C) (1 mg/mL) was added. After 20 min, 2 μL of materials at different concentrations was added in a final volume of 200 μL. The subsequent steps were the same as above for TLR9.

For materials inhibition of TLR4 activation, HEK-Blue hTLR4 reporter cells in DMEM with or without FBS were plated at a density of 2.5×10^4/well in a 96-well plate for 30 min, then 2 μL of

LPS (100 ng/mL) was added. After 20 min, 2 μL of materials at different concentrations was added in a final volume of 200 μL. The subsequent detection steps were same as above.

Anti-inflammatory assays *in vitro*

RAW 264.7 macrophages were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were seeded at 2×10^4 cells/well in a 96-well plate for 30 min, then 2 μL of CpG BW006 (1 mg/mL) was added. After 20 min, 2 μL of materials at different concentrations were introduced in a final volume of 200 μL. After a 24 h incubation, supernatants were collected and the TNF-α level was measured by ELISA.

Cytotoxicity assay

Cytotoxicity of materials was investigated by using a CCK-8 assay. Briefly, RAW 264.7 cells were seeded in 96-well plates at 8,000 cells/well and were cultured for 24 h at 37 °C, 5% CO_2. After treatment with different materials for another 24 h, the medium was replaced with medium containing 10% CCK-8 (100 μL). Cytotoxicity was measured after 1 h by using plate reader at 492 nm.

Quantitative real-time PCR assay *in vitro*

RAW 264.7 cells were seeded at 5×10^5 cells/well in a 6-well plate for 30 min and CpG BW006 (final concentration, 1 μg/mL) was added. After 20 min, materials were introduced at a final concentration of 100 μg/mL. After 24 h of incubation, total mRNA was exacted and reverse transcribed to cDNA. qPCR was performed using a SuperReal PreMix Plus with a qPCR machine (Roche Diagnostics Ltd, Lewes, UK). Amplified transcripts were quantified by the comparative Ct method.

Cellular colocalization of PEI-*g*-ZIF NPs and CpG

RAW 264.7 cells were seeded on coverslips in 12-well culture plates at 5×10^4 cells/well and were incubated for 24 h. The cells were then treated with 1 μg/mL Cy5.5-labeled CpG 1826. After 30 min incubation, the cells were washed with PBS and exposed to FITC-labeled NPs at 100 μg/mL for another 12 h. After staining with DAPI and Lysotracker Red DNA-99, the coverslips were

washed in place and mounted on slides. The samples were imaged using a Nikon structured illumination microscope.

CLP model

C57 mice (male, 6- or 8-week old) were obtained from Liaoning Changsheng Biotechnology. All animal experiments were performed following the guidelines for laboratory animals established by the Animal Care and Use Committee of Northeast Normal University. CLP-induced sepsis was established according to a previously described procedure. Briefly, mice were anesthetized with isoflurane anesthesia, and the abdominal hair was shaved, followed by disinfection of the abdominal area with alcohol prep pads. Then a midline 1-cm incision was made, and the cecum was gently removed from the abdominal cavity, leaving the remainder of the small and large bowel within the peritoneal cavity. After ligation with 4-0 silk at the designated position for severe-grade sepsis, the cecum was punctured with a 21-gauge needle and the cecal contents were extruded through the perforation. The cecum was gently placed back into the peritoneal cavity and the incision was stitched and closed. The mice were divided into five groups and subjected to the different treatments. Sham mice had only the abdominal laparotomy procedure without cecum ligation and puncture. The untreated CLP group underwent the procedure as described above without further treatment. In the PEI 1800-*g*-ZIF NPs and free PEI 1800 treatment groups, PEI 1800-*g*-ZIF NPs or free PEI 1800 (10 mg/kg) were administered at 1 h and 12 h after CLP, and the survival rate, clinical scores, and body weight were monitored for 5 days. The criteria for clinical score were as follows: 0, no symptoms; 1, piloerection and huddling; 2, piloerection, diarrhea, and huddling; 3, lack of interest in surroundings and severe diarrhea; 4, decreased movement and listless appearance; and 5, loss of self-righting reflex. Mice were killed humanely when they exhibited a score of 5.

***Ex vivo* fluorescence imaging**

Mice were intraperitoneally injected with Cy7-labeled cationic NPs at a dose of 10 mg/kg at 1 h post-CLP. The major organs including heart, lung, liver, spleen, and kidney, and the cecum were excised and imaged at 2 h, 12 h, and 24 h post-CLP.

Ratio of M1 polarization macrophages in peritoneal cavity

Flow cytometry was used to quantitatively determine the ratio of M1 polarized macrophages in the peritoneal cavity. Peritoneal lavages were performed with 5 mL of PBS containing 10% FBS, and the collected cells were centrifuged and resuspended in PBS. The cells were then stained with anti-CD11b-FITC, anti-CD11c-PE, and anti-F4/80-PE-Cy7 antibodies at 4 °C for 1 h. After repeated washing with PBS, the cells were analyzed by FACS.

Ratio of cells with high ROS level in peritoneal cavity

Flow cytometry was also used to determine the percentage of cells in peritoneal cavity with high ROS levels. Peritoneal lavages were performed with 5 mL of PBS containing 10% FBS, and the collected cells were centrifuged and resuspended in DMEM without FBS. The cells were then stained for ROS at 4 °C for 2 h. After repeated washing with PBS, the cells were analyzed by FACS.

Extraction and quantification of cfDNA

A QIAamp DNA Blood Mini Kit was used to extract cfDNA from serum and peritoneal lavage fluid. The concentration of cfDNA was determined by using a Quant-iT PicoGreen dsDNA Assay Kit.

Activation of TLR9/MyD88/NF-kB pathway by serum from treated mice

HEK-Blue hTLR9 reporter cells in DMEM without FBS were plated at 8×10^4 cells/well in a 96-well plate for 30 min then were treated 10 μL of serum. After 24 h of incubation, supernatants were collected and were incubated with QUANTI-Blue. Activation of the TLR9/MyD88/NF-kB pathway was determined by SEAP activity by measuring the OD at 620 nm using a plate reader.

Results and Discussion

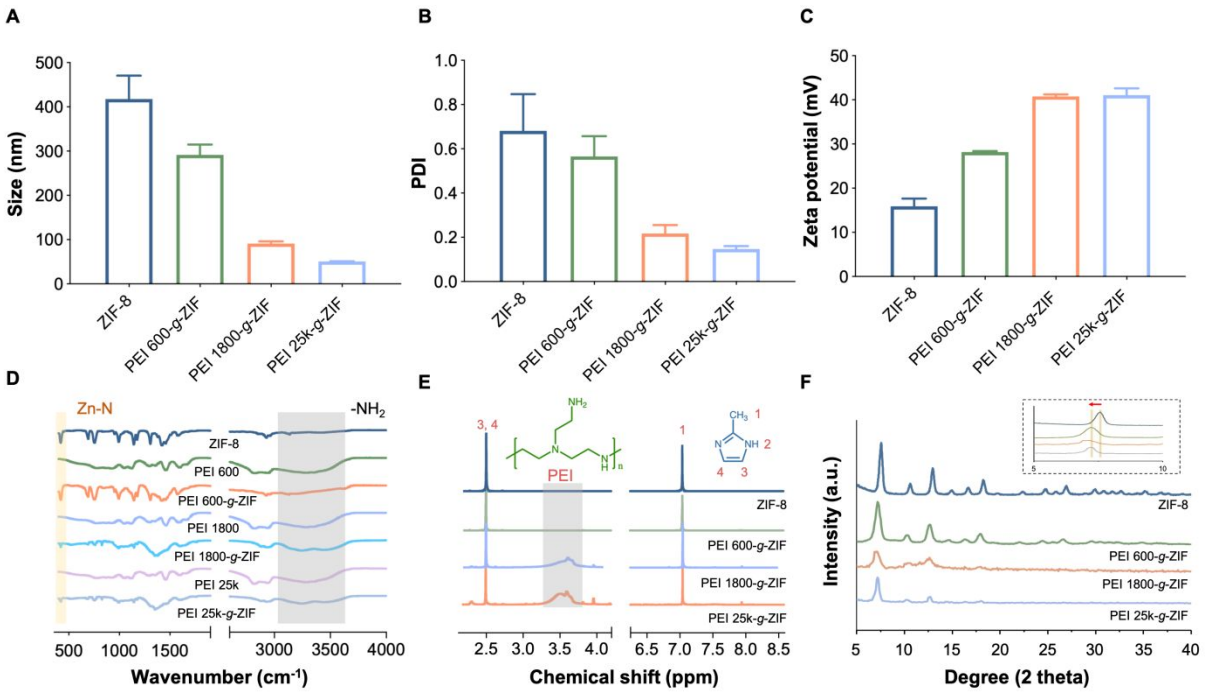

Figure S1. Characterization of NPs. (A) Size, (B) PDI, and (C) zeta potential of pure ZIF-8 NPs and PEI-*g*-ZIF NPs fabricated using PEI of different molecular weights (600, 1800, and 25k Da). (D) FTIR spectra, (E) ^{1}H NMR spectra, and (F) XRD spectra.

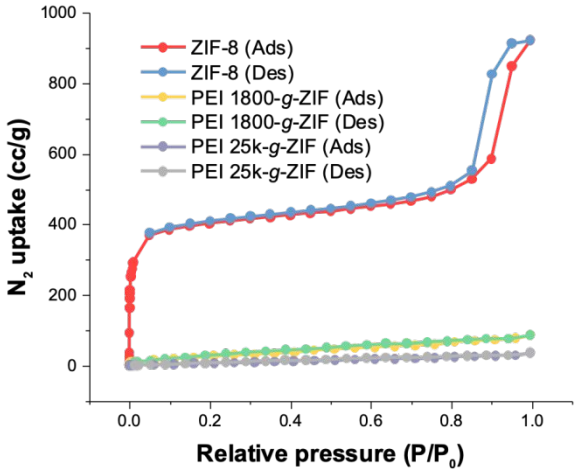

Figure S2. BET surface area result of NPs.

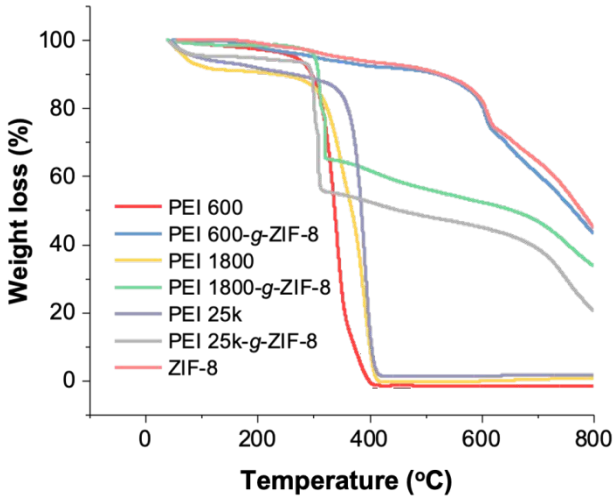

Figure S3. TGA analysis of NPs.

TGA analysis was performed to quantitatively characterize the amount of grafted PEI in the PEI-*g*-ZIF NPs. As shown in Figure S3, degradation of ZIF-8 started at above 600 °C, consistent with previously reported literature. Significant weight loss of the PEI-*g*-ZIF NPs from 250 to 400 °C could be attributed to the decomposition of PEI, indicating the presence of PEI in the NPs. The amount of PEI in the NPs was obtained by subtracting the weight loss between 270 and 390 °C of PEI-*g*-ZIF NPs from that of ZIF-8. There was a 37.3 wt% of PEI 1800 in the PEI 1800-*g*-ZIF NPs, and a 31.5 wt% of PEI 25k in the PEI 25K-*g*-ZIF NPs, no detected amount of PEI 600 was calculated from TGA result, confirming the fact that limited PEI 600 in PEI 600-g-ZIF NPs.

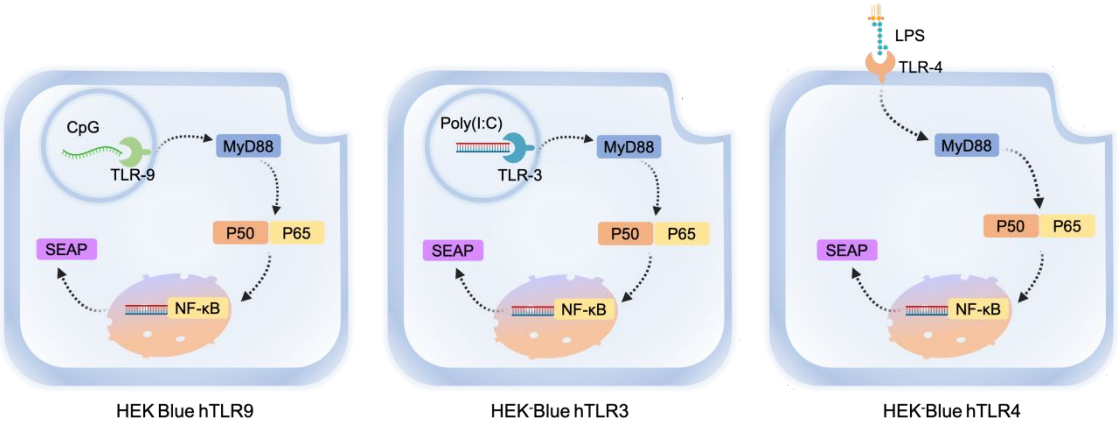

Figure S4. Schematic of activation of HEK-Blue hTLR reporter cells by agonists.

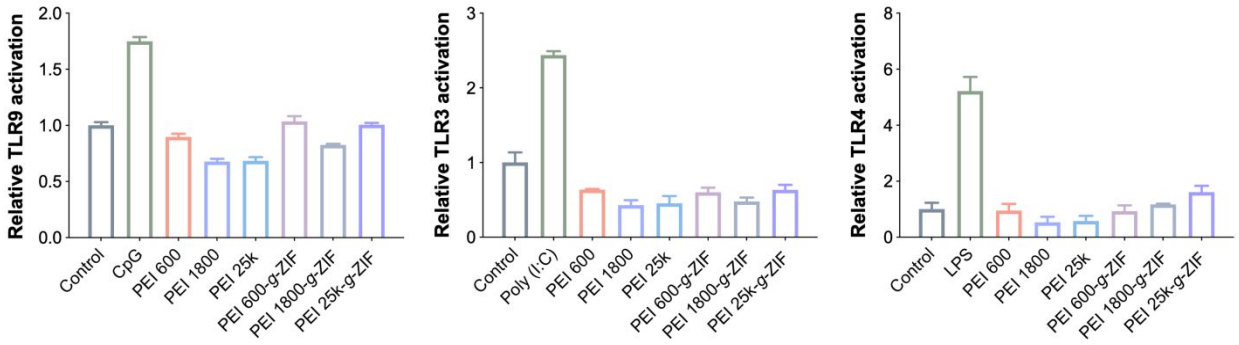

Figure S5. Activation of HEK-Blue hTLR9, hTLR3, and hTLR4 reporter cells by materials (no agonists added).

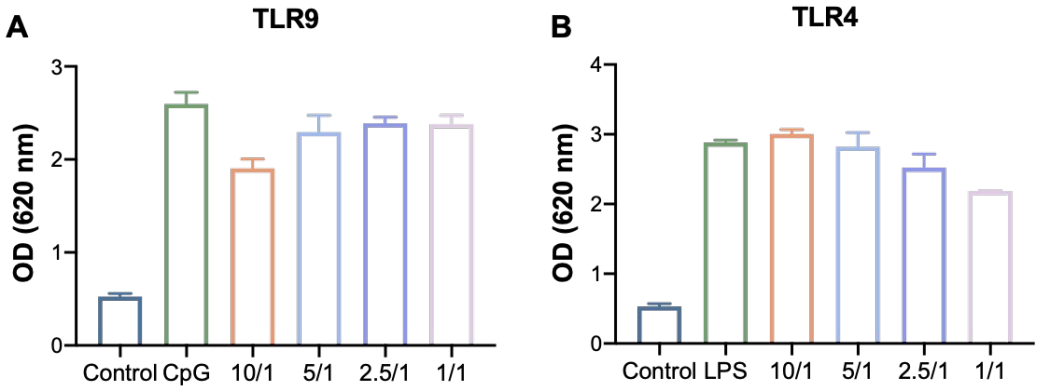

Figure S6. Activation of (A) HEK-Blue hTLR9 and (B) hTLR4 cells after treated by ZIF-8 with different weight ratio to agonists.

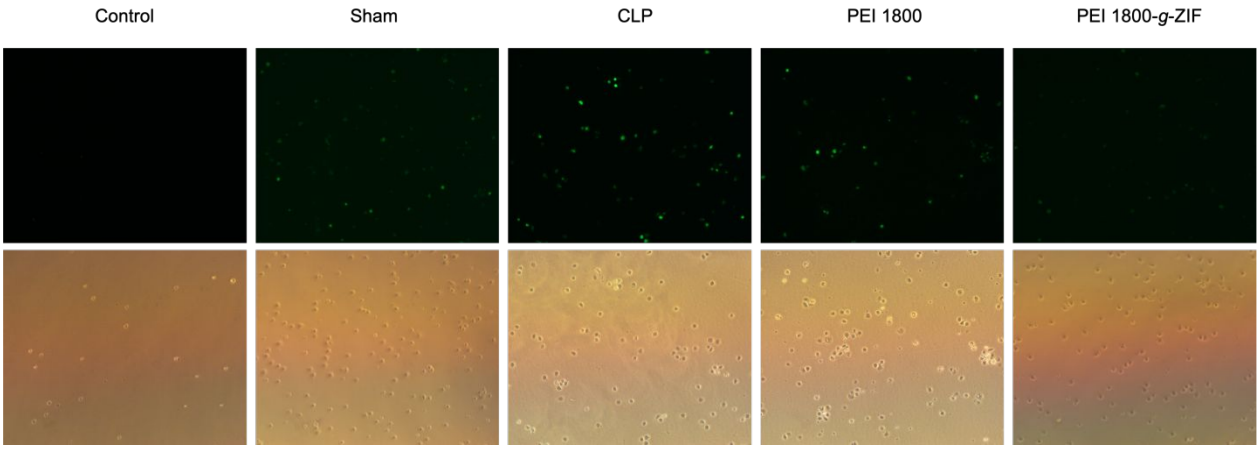

Figure S7. ROS level of cells from peritoneal cavity of mice in different treatment groups.

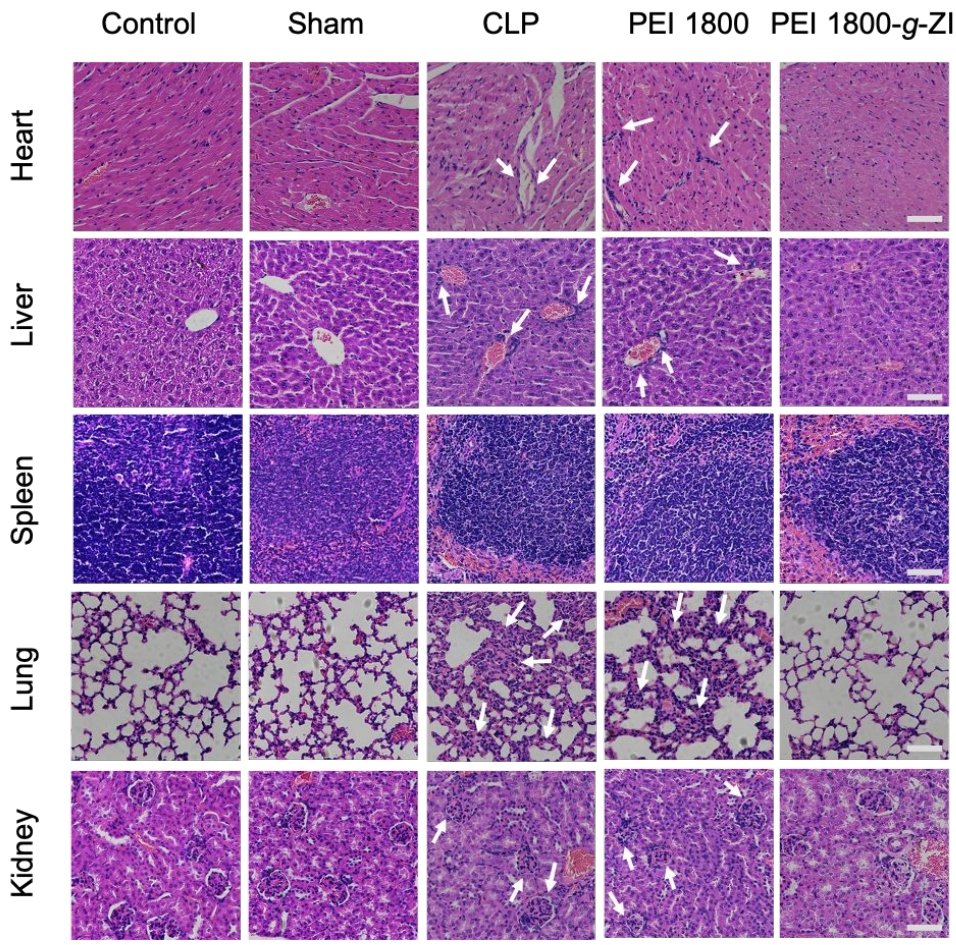

Figure S8. H&E staining images of major organs of mice in different treatment groups. Scale bar, 100 μm.

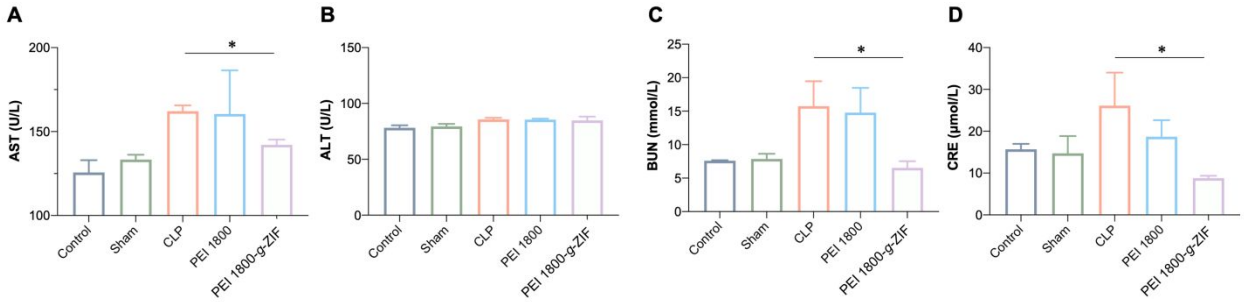

Figure S9. Blood biochemistry analysis of mice in different treatment groups.

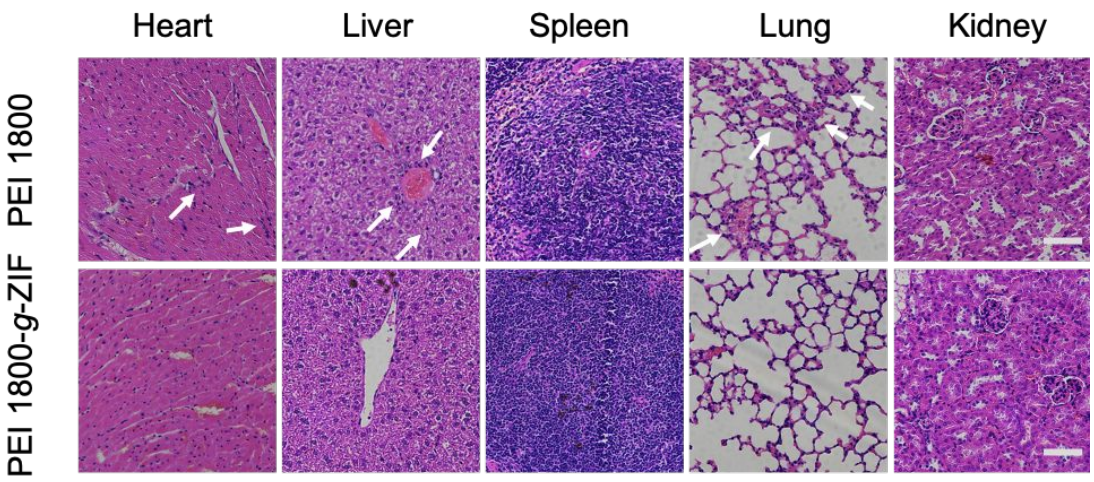

Figure S10. H&E staining images of major organs of mice after injection with free PEI 1800 and PEI 1800-*g*-ZIF NPs for 5 days. Scale bar, 100 μm.

Table S1. Zn content analysis of ZIF-8 and PEI-*g*-ZIF NPs.

	ZIF-8	PEI 600-*g*-ZIF	PEI 1800-*g*-ZIF	PEI 25k-*g*-ZIF
Zn (%)	29.50	22.15	18.24	17.15