Supporting Information

Infected Wound Repair with an Ultrasound-enhanced Nanozyme Hydrogel Scaffold

Fan Zhang[1,6,7#], Yong Kang[2,#], Liwen Feng[3], Guan Xi[1,6,7], Wei Chen[5], Na Kong[5], Wei Tao[5,*], Tiangang Luan[1,6*], Seyoung Koo[5,*], Xiaoyuan Ji[2,4,*]

[1] School of Biomedical and Pharmaceutical Sciences, Guangdong University of Technology, Guangzhou 510630, PR China

[2] Academy of Medical Engineering and Translational Medicine, Medical College, Tianjin University, Tianjin, 300072, China

[3] Boji Pharmaceutical Research Center, Boji Medical Biotechnological Co. Ltd., Guangzhou 510630, PR China

[4] Medical College, Linyi University, Linyi 276000, China

[5] Center for Nanomedicine and Department of Anesthesiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115, USA

[6] Jieyang Branch of Chemistry and Chemical Engineering Guangdong Laboratory (Rongjiang Laboratory), Jieyang 515200, China

[7] Smart Medical Innovation Technology Center, Guangdong University of Technology, Guangzhou 510630, PR China

*Corresponding authors:

wtao@bwh.harvard.edu (W. Tao), cesltg@gdut.edu.cn (T. Luan), hs8019@uhs.ac.kr (S.Koo), jixiaoyuan@tju.edu.cn (X. Ji)

Methods

Synthesis of imine-traizine-benylpyridine derivative ligand

2,4,6-Tris(4-aminophenyl)-1,3,5-triazine (500 mg, 1.41 mmol), 4-pyridylaldehyde (1.32 mL, 14.1 mmol) and trifluoroacetic acid TFA (0.02 mL, 0.2 mmol) were dissolved in THF under an argon atmosphere. The mixture was stirred at reflux for 72 hours. Sodium bicarbonate (89 mg, 0.85 mmol) was added into the resulting mixture for neutralization. After filtration, the filtrate was evaporated under reduced pressure. The crude product was washed with diethyl ether and recrystallized from a THF/diethyl ether mixture (9/1), affording a yellow solid (780 mg, 89%). The ^{1}H-NMR spectra in solution (400 MHz, THF-d_8, 25 ℃): ^{1}H NMR δ = 8.9 (dd, *J* = 6.2 Hz, 2.1 Hz, 6H, PhH), 8.75 (dd, *J* = 4.4 Hz, 1.6 Hz, 6H, PhH), 8.68 (s, 3H, CH=N), 7.85 (dd, *J* = 6.2 Hz, 2.1 Hz, 6H, PhH), 7.48 (dd, *J* = 6.2 Hz, 2.1 Hz, 6H, PhH) ppm.

Preparation and Characterization of PNAs

imine-traizine-benylpyridine derivative (12.4 mg, 0.02 mmol) and terephthalic acid (4.98 mg, 0.03 mmol) were dissolved in 5 mL of DMF solution, and potassium tetrachloroplatinate (24.9 mg, 0.06 mmol) was dissolved in 5 mL deionized water. The mixture was poured into a stainless-steel reactor and kept in a 100 °C oven for 12 h. The precipitates were collected by centrifugation (10000 rpm/min) and washed with DMF and water three times. The resulting black solids were dried under reduced pressure to give the final product.

The morphologies of the PNAs were characterized by transmission electron microscopy (TEM) using a JEOL microscope (JEM-2100F, JEOL, Japan) at an accelerating voltage of 200 kV. The surface morphologies and elemental distributions were analyzed by scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM–EDS, Hitachi SU8010). The hydrodynamic diameters and zeta potential were measured by DLS (Zetasizer Nano ZS90, Malvern Instruments, UK). The original features of the PNAs were determined by X-ray photoelectron spectroscopy (XPS, Axis HSi, Kratos Ltd., UK) and inductively coupled plasma atomic emission spectrometry (ICP–AES).

CAT-like activity of PNAs

The CAT-like activity of the PNAs was evaluated by a dissolved oxygen (DO) meter (LEICI JPSJ-605F). The produced O_2 (mg L^{-1}) from the addition of H_2O_2 (1, 2, 3, 4, 5, 6 mM) was quantified in the deoxygenated water of PNAs (2 μg mL^{-1}) (n = 3 for each group).

POD-like activity of PNAs

The POD-like activity assay of the PNAs was carried out using H_2O_2 or TMB as the POD substrate. In a typical test, TMB (0.2 mg mL^{-1}), H_2O_2 (4 mM), and PNAs (25, 50, 75, 100, 125, 150, 200 μM Pt, 25 μM Pt was equal to 11.1 μg mL^{-1}) or HRP (0.005, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04 U mL^{-1}) were mixed at room temperature in an HAc-NAc buffer solution (pH = 5.5). In the case of H_2O_2 as the substrate, PNAs (25 μM Pt) and TMB (0.2 mg mL^{-1}) were added into the HAc-NAc buffer solution (pH = 5.5) at various concentrations of H_2O_2 (2, 4, 8,10, 20, 40, 80 mM). In the case of TMB as the substrate, PNAs (25 μM Pt) and H_2O_2 (10 mM) were added into the HAc-NAc buffer solution (pH = 5.5) at various concentrations of TMB (0.01, 0.02, 0.04, 0.08, 0.1 mg mL^{-1}). The absorbance changes of the reaction mixture at 652 nm were measured in time-scanning mode to assess the POD-like activity (n = 3 for each group).

GR-like activity of PNAs

The GR-like activity assay of the PNAs was carried out using GSSG as the GR substrate and NADPH as the GR coenzyme. In a typical test, GSSG (500 μM) and PNAs (100 μg mL^{-1}) were reacted for 5 mins at room temperature in a GSH/GSSG assay kit buffer solution (Beyotime S0053). Then, DTNB (3 mg mL^{-1}) and NADPH (0.5 mg mL^{-1}) were added to the reaction mixture. The ultrasound group was given at a frequency of 1 MHz, 0.5 W cm^{-2} for 5 mins. The absorbance changes of the reaction mixture at 412 nm were measured in time-scanning mode to assess the GR-like activity (n = 3 for each group). The DTNB and GSH derivatization product GS-TNB was detected at the characteristic molecular ion peak of m/z 503.06 to determine the presence of GSH. The GS-TNB in the positive control group was obtained by adding GSH (0.1 μmol) to the solution of DTNB (0.05 μmol). The high-resolution mass spectrometry determination conditions

were as follows: ion source: HESI (negative mode); spray voltage: 3 kV; dry dryness: N_2; drying gas temperature: 350 °C.

Method for calculating the initial reaction rate

The initial reaction rate was determined following the method reported. According to the Lambert–Beer law (Eq. (1)), we can derive it as follows (Eq. (2) (3)):

$$A=e\cdot L\cdot c \quad (1)$$

$$c=A/(e\ L) \quad (2)$$

$$(d[c])/(d[t])=(d[A])/(d[t]) \quad 1/(e\ L) \quad (3)$$

where d[c]/d[t] is the initial velocity (v) and d[A]/d[t] are obtained by calculating the slopes (k) of the initial absorbance changes with time. Therefore, Eq. (3) can be replaced by Eq. (4).

$$v=k/(e\ L) \quad (4)$$

where e is the molar absorption coefficient for TMB-derived oxidation products (39000 $M^{-1}\ cm^{-1}$) and L is 1 cm.

The reaction times of the CAT-nanozyme and POD-nanozyme PNAs were determined first with H_2O_2 or TMB as the substrate. Based on the data of Figure 3a, 3i, we confirmed that absorbance determination was performed in 300 s for PNAs CAT-nanozyme (2 μg mL^{-1}PNAs) and 600 s for PNAs POD-nanozyme (11.1 μg mL^{-1} PNAs).

Method for calculation of apparent kinetic parameters

Based on the data of initial velocity (Fig. 2c, 2 g and 2k) and the concentrations of substrate, the apparent kinetic parameters of PNAs were obtained from the Michaelis–Menten equation.

ROS Scavenging Capacity

The ROS scavenging capacity of PNAs was evaluated using H_2O_2 as a representative ROS. The H_2O_2 scavenging capacity was determined by mixing 10 mL of 1 mM H_2O_2. The ultrasound group was given at a frequency of 1 MHz, 0.5 W cm^{-2} for 5 mins.

Cell Culture

L929 cells, HaCaTs, and HUVECs were purchased from ATCC. Human skin fibroblasts (HSFs) were cultured in Dulbecco's modified Eagle’s medium (DMEM; Gibco) enriched with 10% fetal bovine serum (FBS). In parallel, human umbilical vein

endothelial cells (HUVECs) and HaCaT cells were maintained in RPMI 1640 medium (Gibco) with 10% FBS.

Intracellular ROS Scavenging Determination

The capacity of the samples to scavenge ROS was assessed using the 2',7'-dichlorofluorescin diacetate (DCFH-DA) probe. H_2O_2, and the samples were introduced into a 6-well plate, which housed cultured cells. Following a 12 h incubation period, the DCFH-DA probe was added and further incubated for 20 minutes. Subsequently, fluorescence microscopy was employed to capture images of various experimental groups.

Cell Toxicity and Migration

To evaluate cell toxicity and mobility, L929 cells, HUVECs, and HaCaTs were employed. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to assess the cytotoxicity of the PNAs. For this, cells were assigned to various treatment conditions and positioned atop the cultured cells. In the ultrasound-treated group, the samples were exposed to 1 MHz, 0.5 W cm^{-2} ultrasound for 5 minutes. On day 2, the MTT solution was added to each well and incubated for 2 hours, followed by the measurement of absorbance at 490 nm. A scratch assay with a 100 μL pipette was performed to evaluate cell migration under different treatments. Photographic documentation of cell migration was undertaken 24 hours posttreatment.

Tube Formation Assay

A total of 250 μL of thawed Matrigel (BD, USA) was added to each lower chamber of a 24-well plate to investigate tube formation. After incubation at 37 °C for 1 h, 5×10^4 HUVECs were seeded into the Matrigel-coated lower chamber and exposed to various treatments. In the ultrasound group, the sample was exposed to 1 MHz, 0.5 W cm^{-2} for 5 min. A microscope recorded the HUVEC tube images, and ImageJ software was used to evaluate tube formation.

Anti-Inflammatory Capacity

The anti-inflammatory macrophage model induced by LPS. After 12 h of LPS stimulation, macrophages were cultured in hydrogel extracts. Macrophages were fixed with 4% paraformaldehyde after 24 h, and immunofluorescence staining for CD206

and CD86 was performed to evaluate the anti-inflammatory capability.

Infected wound healing

To assess the therapeutic effect of PNAs embedded within gelatin methacryloyl hydrogel (GelMA+PNAs), a mouse model of infected wounds was established. All protocols of animal experiments were duly approved by the Institute Animal Ethics Committee at Tianjin University. For the experiment, mice with a weight range of 25–30 g were intramuscularly administered streptozotocin (40 mg/kg). After 3 weeks, mice with blood glucose levels exceeding 16.1 mmol/L in two consecutive readings were identified as simulative diabetic. These mice were then anesthetized, and their dorsal fur was fully removed. Subsequently, full-thickness wounds with a diameter of 10 mm were created using skin biopsy punches, followed by the inoculation of a 10 μL *S. aureus* suspension (1×10^7 CFU/mL) to establish an infected wound model. One day after infection, bacterial colony counts escalated from 10^5 to 10^6 CFU, verifying the successful establishment of the infection model. Individual wounds were treated with GelMA, GelMA+PNAs, and GelMA+PNAs with ultrasound (1 MHz, 0.5 W cm^{-2}, 5 min). Wound photographic documentation and tissue collection for histological examination were conducted on days 3, 5, 7, and 9. Hematoxylin and eosin (H&E) and Masson staining were performed on days 3 and 9. On day 6, skin was excised to determine hypoxia level, reactive oxygen species (ROS) level, vascularization, collagen deposition, epidermal formation, and macrophage polarization through hypoxia-inducible factor-α (HIF-α), dihydroethidium (DHE) probe, CD31/α-smooth muscle actin (α-SMA), collagen/vimentin, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and CD206/CD86 immunofluorescence staining, respectively. On day 8, major organs such as the heart, liver, spleen, lungs, and kidneys were harvested, and H&E staining was conducted to assess the biocompatibility and biosafety of hydroxyethyl acrylate (HEA)@Gel-based treatments.

Statistical analysis

Statistical analysis was performed using the Student's t-test with statistical significance assigned at *$P < 0.05$ (significant), **$P < 0.01$ (moderately significant), ***$P < 0.001$ (highly significant), and ****$P < 0.0001$ (extremely significant).

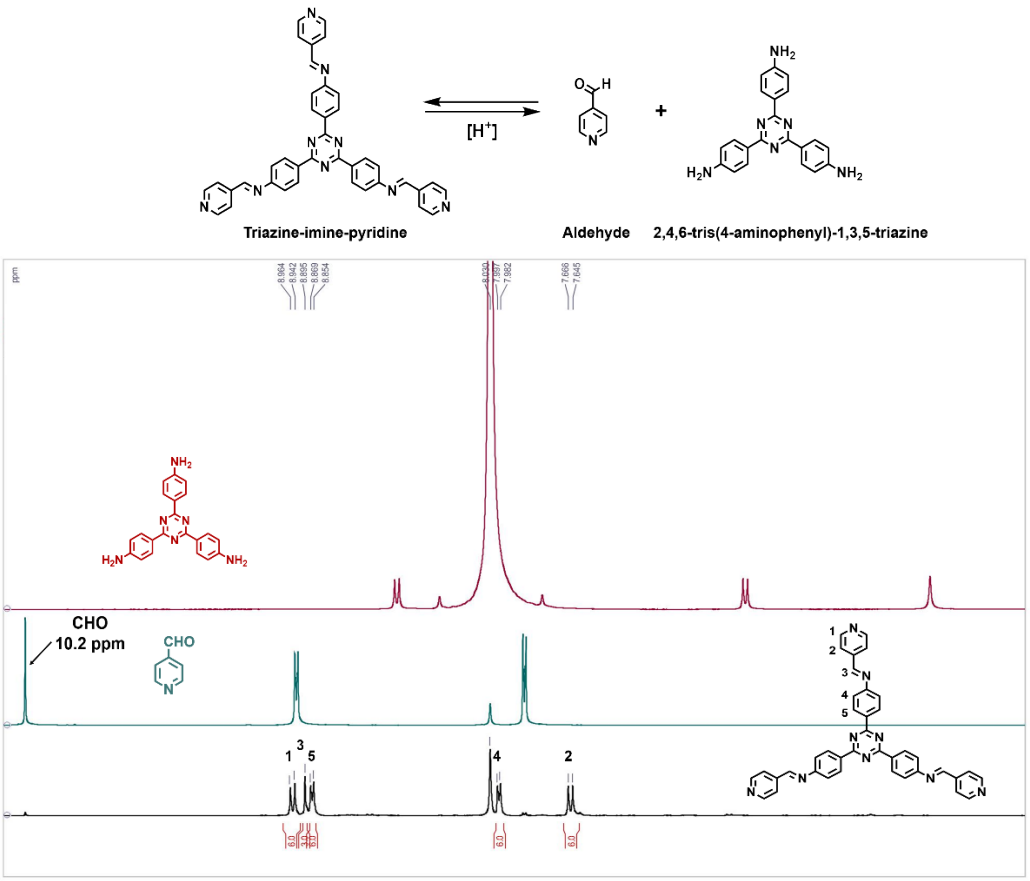

Figure S1. The ^{1}H NMR spectra of the imine-triazinebenylpyridine ligand, 4-pyridinecarboxaldehyde and 2,4,6-tris(4-aminophenyl)1,3,5-triazine in solution (DMF-d_7, 25 °C).

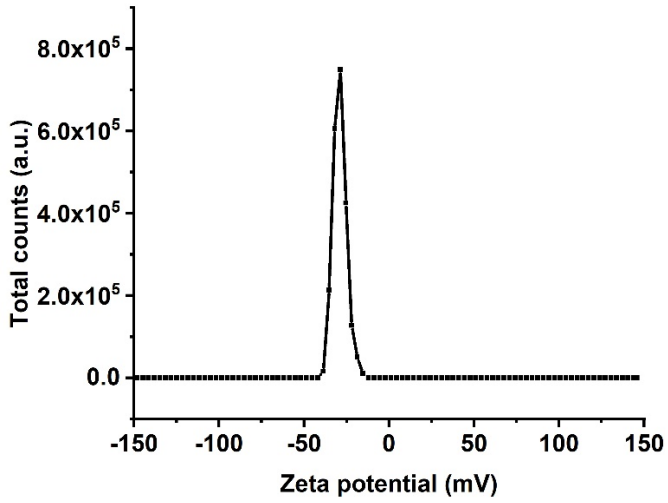

Figure S2. Zeta potential of PNAs.

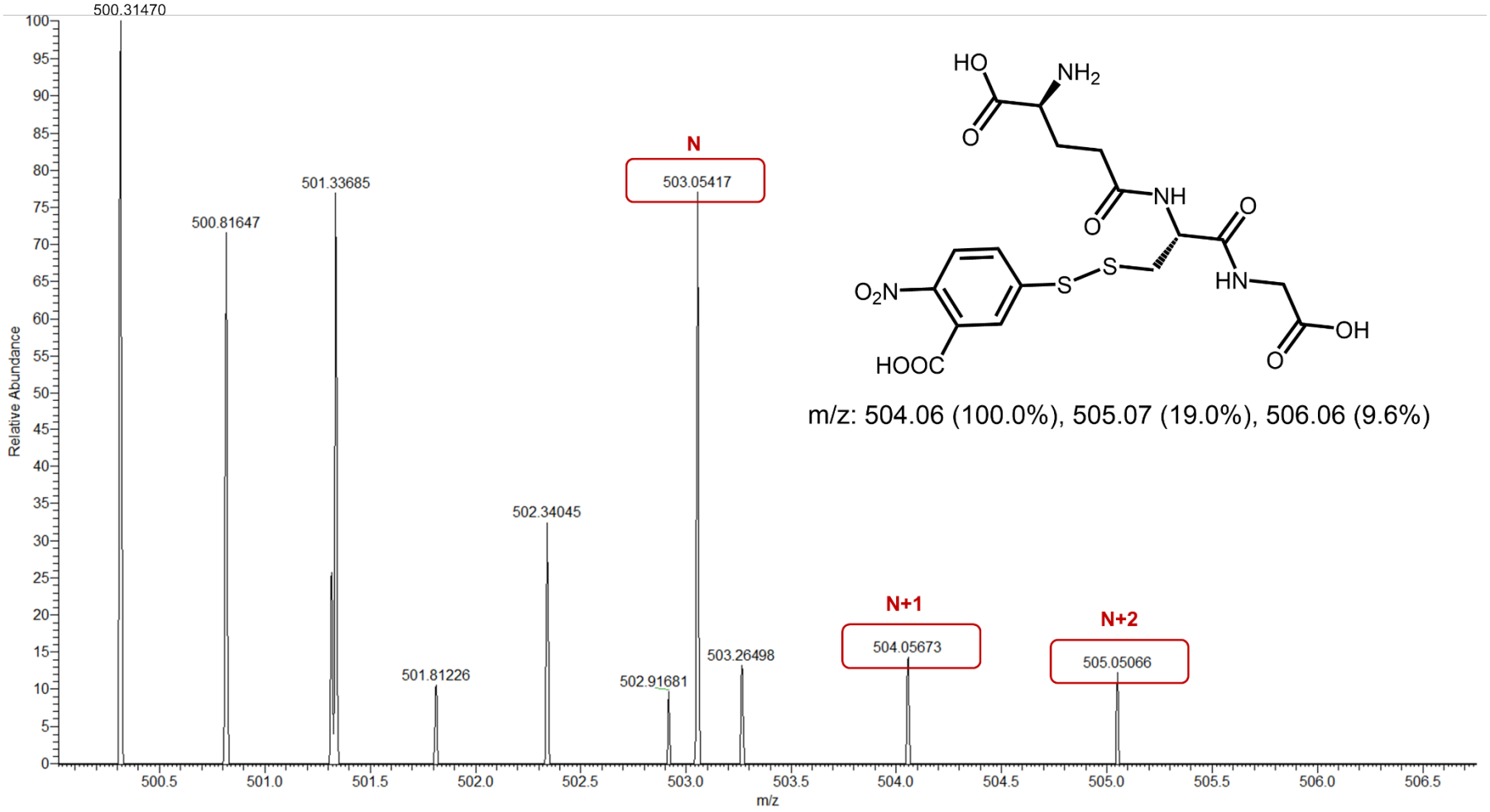

Figure S3. ESI-MS spectrum (negative mode) of GS-TNB in the positive control group (GSH and DTNB in buffer solution).

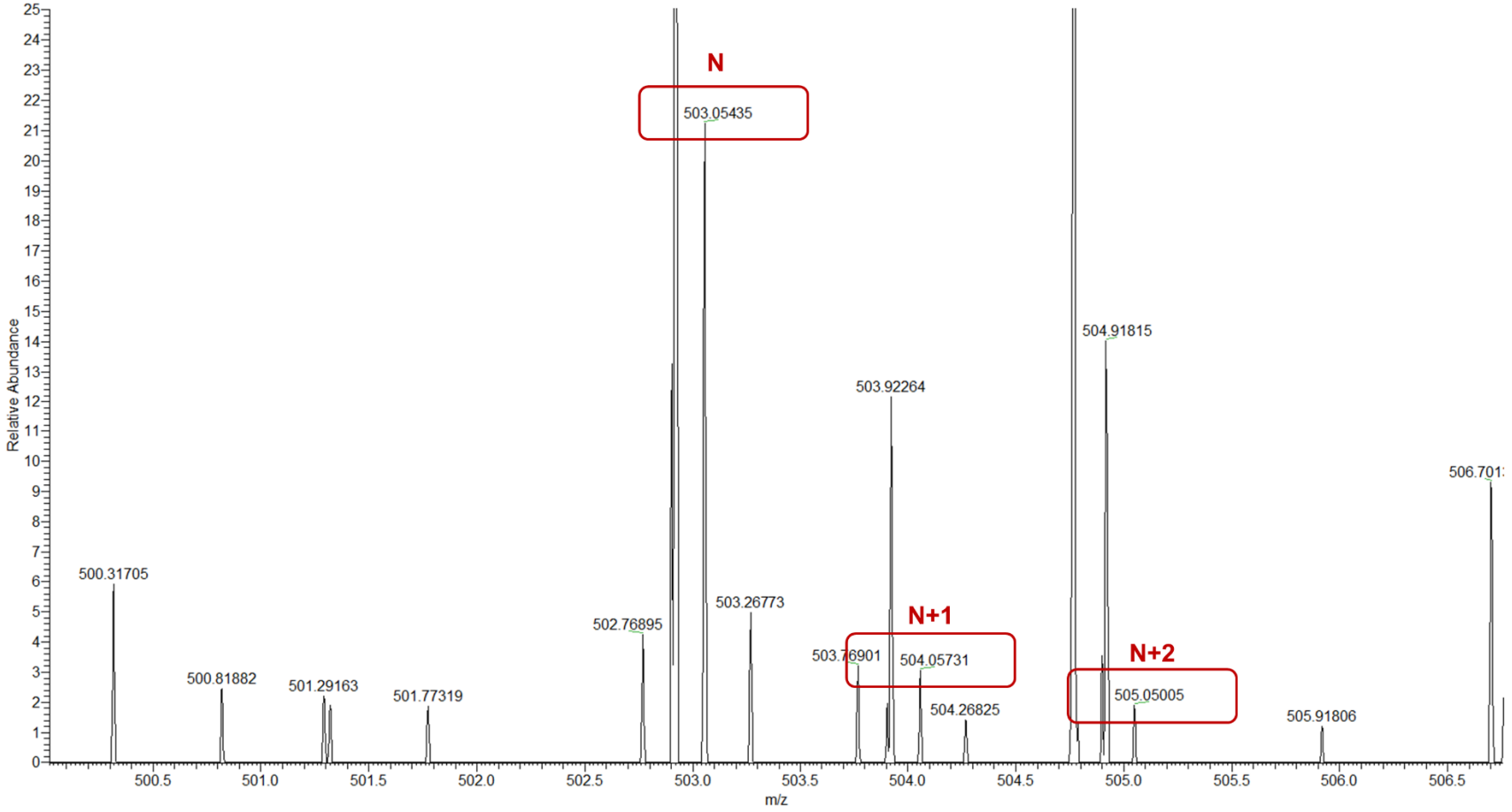

Figure S4. The ESI-MS spectrum (negative mode) of GS-TNB in PNAs+ultrasound group (GSSG, DTNB, NADPH, and PNAs in the buffer solution with 1 MHz, 0.5 W cm^{-2}, 5 mins).

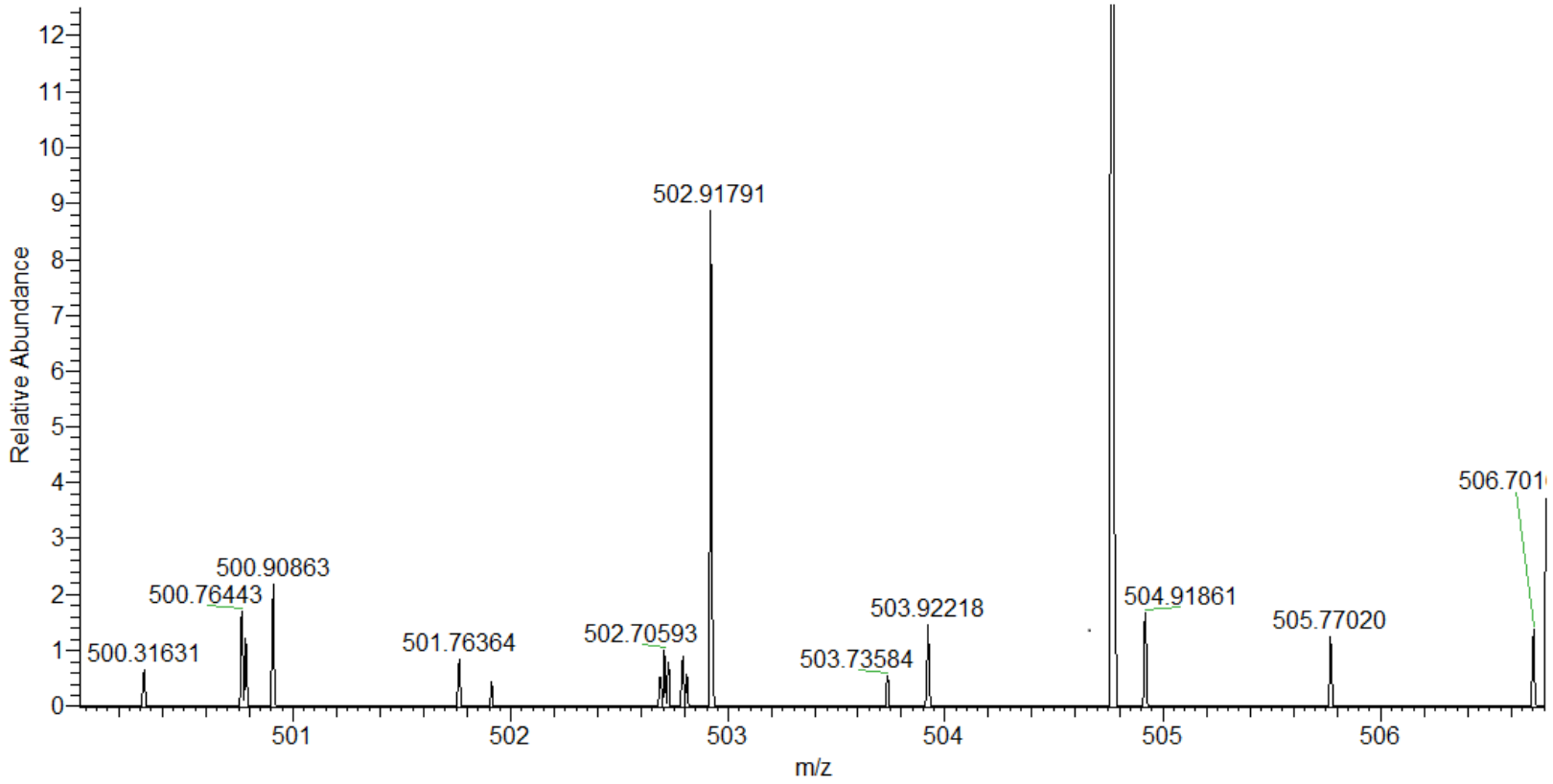

Figure S5. The ESI-MS spectrum (negative mode) of GS-TNB in control group (GSSG, DTNB, and NADPH in buffer solution).

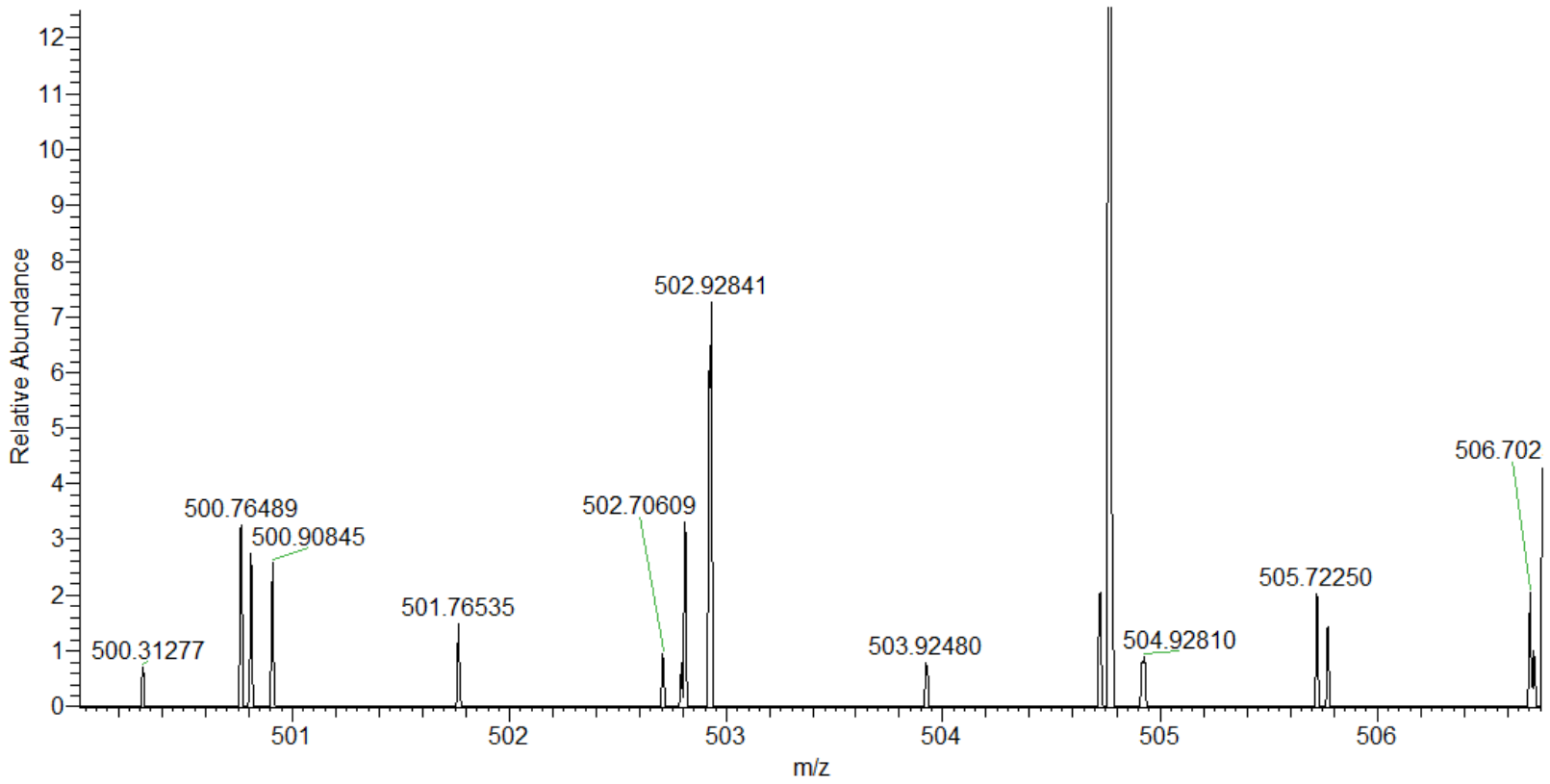

Figure S6. The ESI-MS spectrum (negative mode) of GS-TNB in ultrasound group (GSSG, DTNB, and NADPH in the buffer solution with 1 MHz, 0.5 W cm^{-2}, 5 mins ultrasound).

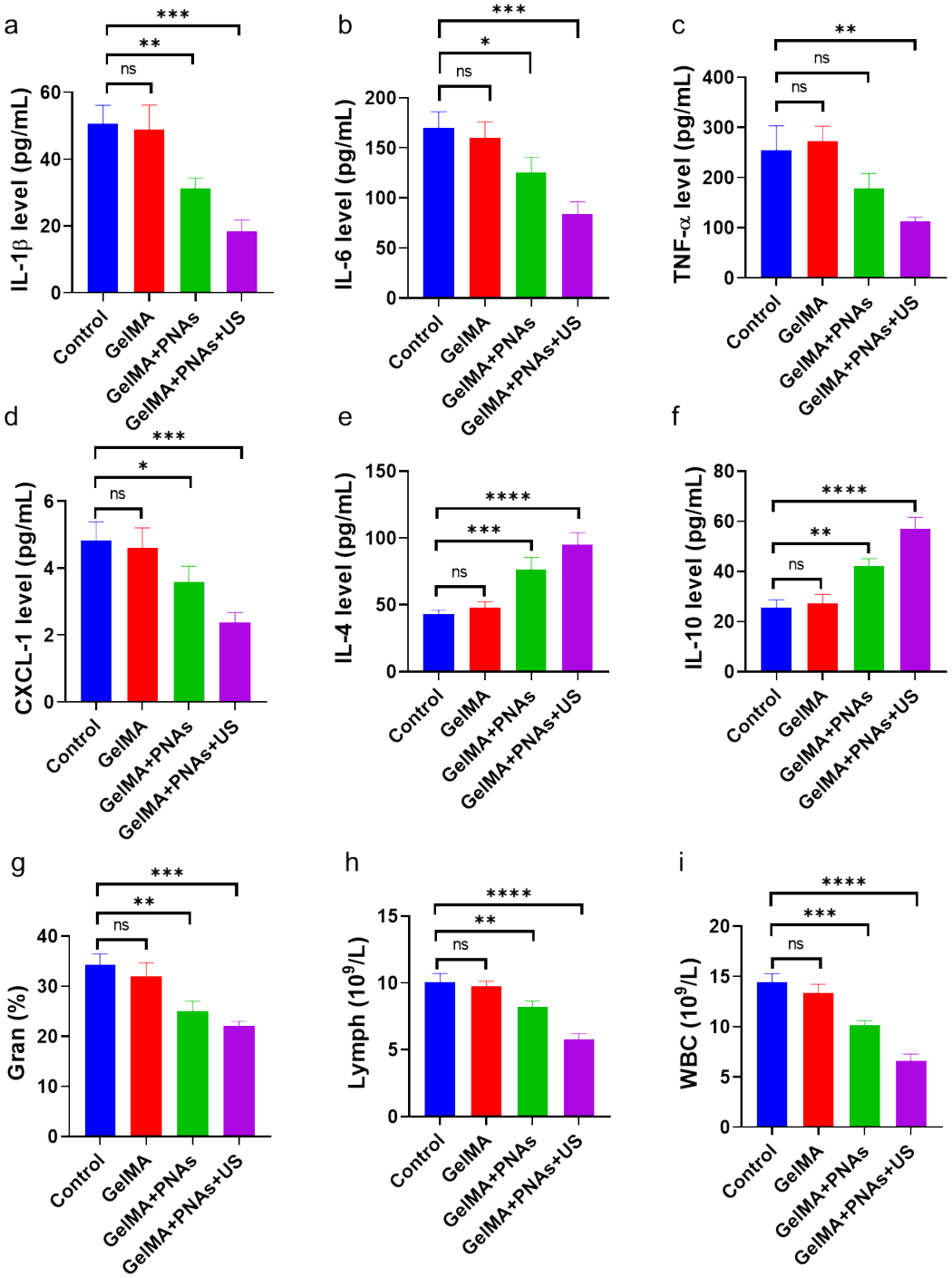

Figure S7. Regulation of the immune microenvironment in vivo. In vivo wound concentrations of (a) IL-1β, (b) IL-6, (c) TNF-α, (d) CXCL-1, (e) IL-4, and (f) IL-10. (g) Neutrophil percentage (gran%), (h) lymphocyte count, and (i) white blood cell count in peripheral blood. Data are presented as the mean ± s.d. (n = 3 independent mice).