Electronic Supplementary Information

for

A membrane-penetrative COF-based nanoplatform for intracellular bacterial pathogens eradication across diverse niches

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Methods.

1. Chemicals

5,10,15,20-Tetrakis(4-aminophenyl) porphyrin (TAPP) and 2,5-dihydroxyterephthalaldehyde (DHA) were purchased from Macklin. Rhodamine B (RhB) was purchased from Sigma-Aldrich. Larginine and phenylmethanesulfonyl fluoride (PMSF) were purchased from Aladdin Reagent (Shanghai, China). TLR2/Toll-like Receptor 2 Rabbit Polyclonal Antibody, TLR4/Toll-like Receptor 4 Rabbit Polyclonal Antibody, FITC-labeled Goat Anti-Rabbit IgG (H+L), 3-Amino,4aminomethyl-2',7'-difluorescein, diacetate (DAF-FM DA), and Reactive Oxygen Species Assay Kit were purchased from Beyotime, BBoxiProbe® Fluorescent probe O56 was purchased from Bestbio.

2. Characterizations

SEM images were recorded on a Hitachi S-4800 FESEM. TEM images were recorded on a FEI Tecnai G2F20 high-resolution transmission electron microscope. The UV-Vis absorption spectra were recorded on a Jasco V-550 UV/Visible spectrophotometer (JASCO, Japan). Fluorescence spectra were recorded on a JASCO FP-6500 spectrofluorometer. Hydrodynamic diameters and ζ potential measurements were detected on Malvern Nano ZS-90 at 25 °C. Powder X-ray diffraction (XRD) measurement was conducted by a BRUKER D8 ADVANCE X-ray diffractometer equipped with CuK α radiation (λ =0.15406 nm). XPS measurements were performed on a Thermo Fisher Scientific ESCALAB 250Xi XPS system. Advantage software was used for further analysis.

3. Synthesis of B-COF

B-COF was synthesized following a previously reported procedure. TAPP for B-COF (0.022 mmol) was dissolved in o-DCB/BuOH (0.5 mL/0.5 mL) in a Pyrex tube (10 mL), followed by the addition of TPA (0.044 mmol) and acetic acid (3 M, 0.1 mL). The mixture was degassed by three freeze–pump–thaw cycles before being sealed off. The tube was then heated at 120 °C for 3 days. The precipitate was separated via centrifuge and washed with THF and acetone. The powder was dried under a vacuum overnight to give the corresponding COF.

4. Synthesis of Mn-TAPP

60 mL of DMF and 3.3 g (4.5 mmol) of TAPP were added to a three-nozzle flask fitted with a

condensing tube and thermometer, stirred to dissolve fully, heated to reflux, then 2.0 g of MnCl₂·4H₂O (10 mmol) was added, and the system was cooled to room temperature after reflux at 135 °C for 2 h. Then, disperse the mixture into 200 mL of deionized water and add ammonia to adjust the pH to 9 at room temperature. The solid product is fully precipitated by resting, pumped, and filtered, and then the collected solid is dried in a vacuum drying oven to obtain the crude product. The pure product was obtained by the chromatographic column, and the obtained product was placed in the vacuum-drying oven to get a dark green solid.

5. Synthesis of Mn-COF

Mn-TAPP (0.022 mmol) was dissolved in o-DCB/BuOH (0.5 mL/0.5 mL) in a Pyrex tube (10 mL), and then TPA (0.044 mmol) and acetic acid (3 M, 0.1 mL) were added. Before sealing, the mixture is degassed through three freezer pump-thawing cycles. It is then heated at 120 °C for 3 days. The precipitate is separated by centrifuge and washed with tetrahydrofuran and acetone. The powder was dried overnight under a vacuum to obtain the corresponding COF.

6. Synthesis of Mn-COF+L-Arg

Mn-COF (1 mg·mL⁻¹, 5 mL) and L-Arg (10 mg·mL⁻¹, 5 mL) were mixed in an aqueous solution. After stirring at room temperature for 24 h, the precipitate was obtained by centrifugation at 12000 rpm for 10 min and then washed with ultra-pure water 3 times. The final product was obtained by centrifugation under the same conditions.

7. Detection of ROS release production in vitro

First, DCFH, which was converted from DCFH-DA, was employed as a probe for ROS measurement. 2 mL of 0.01 M NaOH was added in 0.5 mL of DCFH-DA in DMSO to chemically hydrolyze DCFH-DA to DCFH in the dark for 30 min at room temperature. Then, 10 mL of the Tris buffer (25 mM, pH 7.2) was added to stop the reaction. The stock solution of DCFH was kept on ice in the dark before use. Mn-COF (100 μ g·mL⁻¹) was mixed with DCFH (10 μ M) stock solution. The sonodynamic instrument was then used to ultrasound the mixture for 5 min (1 MHz, 1 W cm⁻², 50 % duty cycle). The fluorescence of the supernatant was measured by centrifugation immediately after ultrasound. The ROS production of Mn-COF at different concentrations was tested in the same

way.

Then, DPBF is also used for singlet oxygen detection. 10 µL DPBF/DMSO solution (10 mM) was added to 1 mL tris buffer containing Mn-COF (100 µg·mL⁻¹). The mixture is then ultrasound at different times. The characteristic UV-vis absorption spectrum of DPBF was determined to determine singlet oxygen generation.

8. Detection of NO production

The NO produced during the reaction was detected by the Griess method (nitrate reductase method). $1mg \cdot mL^{-1}$ Mn-COF+L-Arg and Mn-COF+L-Arg@Sr nanomaterials were incubated with 10 mM H_2O_2 in phosphate-buffered saline (PBS) at 37 °C for 4 h, and Mn-COF was used as the control group. After centrifugation, 50 µL supernatant was taken, and the relative yield of NO was detected with NO assay kit.

9. Cell culture

RAW264.7 mouse macrophages were cultured in an incubator with humidified 5 % CO_2 at 37 °C. The media was Dulbecco's Modified Eagle Medium (DMEM) containing heat-inactivated FBS (10 %).

10. Immunofluorescence imaging of bacteria-stimulated macrophages

RAW264.7 cells were seeded at a density of 1×10^4 cells/well on glass coverslips in 24-well plates. 24 h after seeding, *S. aureus* at a density of 2.5×10^6 CFU per well was added into the cell culture medium for 3 h, then the cells were rinsed with PBS before being fixed in 4 % formaldehyde for 15 minutes. After that, 5 % BSA dissolved in PBS was utilized to block the cells at 37 °C for 1 h. Subsequently, the macrophage cells were incubated with polyclonal rabbit anti-TLR-2 or anti-TLR-4 antibody (Beyotime Biotechnology, 1:100) overnight at 4 °C, followed by incubation with FITCconjugated-goat anti-rabbit antibodies (Beyotime Biotechnology 1:200) for 1 h at 37°C. After being washed with PBS, the cells were counterstained with DAPI for another 10 minutes and imaged by a confocal microscope (Nikon A1R).

11. Synthesis of bacteria-stimulated macrophage membrane fragments

RAW264.7 cells were seeded at a density of 1×10^5 cells/well on glass coverslips in 6-well plates. 24 h after seeding, *S. aureus* at a density of 2.5×10^7 CFU per well was added into the cell culture medium for 3 h. Subsequently, the cells were washed twice with PBS. Cells were further separated with a cell scraper and centrifuged at 700 g for 5 min. The collected cells were resuspended in precooled PBS buffer (pH=7.4) and 3 centrifuged again at 600 g for 5 min. The obtained cell pellets were suspended in a hypotonic lysing buffer containing membrane protein extraction reagent (Beyotime Biotechnology) and PMSF and incubated in an ice bath for 10-15 min, based on the manufacturer's instructions. After that, the cells in the above solution were broken repeatedly using a freeze-thaw method followed by centrifugation at 700 g for 10 min at 4 °C. The supernatant was subjected to further centrifugation at 14000 g for 30 min to collect the cell membrane fragments. The membrane products were lyophilized overnight, weighed, and stored at -80 °C. The lyophilized membrane materials are rehydrated in UP water or PBS buffer (pH=7.4) before use.¹

12. Synthesis of macrophage membrane-coated Mn-COF+L-Arg

To construct macrophage membrane-coated Mn-COF+L-Arg, a successive extrusion method was established. In brief, the Mn-COF+L-Arg with a concentration of 100 μ g·mL⁻¹ as prepared above was mixed with the above macrophage membrane fragments in PBS. Afterward, the mixture was transferred into a syringe and extruded through a 450 nm membrane filter and then centrifuged at 12,000 rpm for 10 min to remove excess macrophage membranes. The macrophage membrane coated Mn-COF+L-Arg was rinsed with PBS and stored at 4 °C for further use.

13. Preparation of RhB-labelled Mn-COF+L-Arg@Sr

RhB and Mn-COF+L-Arg with a mass ratio of 5:1 were dispersed in a mixture solvent of ethanol and water (1:1 v/v) and stirred at room temperature for 24 h in the dark for RhB loading. The products were washed with UP water and re-dispersed, resulting in RhB-labelled Mn-COF+L-Arg. The macrophage membrane fragments were coated on RhB-labelled Mn-COF+L-Arg according to step 12, resulting in RhB-labelled Mn-COF+L-Arg@Sr.

14. Cell viability study

The cell viabilities were determined by a standard MTT assay. Briefly, RAW264.7 macrophages

 $(1 \times 10^4 \text{ cells/well})$ were seeded in 96-well plates and incubated overnight. After incubating with Mn-COF+L-Arg@Sr for 24 h, MTT was added to the culture medium and incubated for 4 h at 37 °C in 5 % CO₂. Then, 100 µL of DMSO was added to dissolve the formazan crystals, and a microplate reader (Bio-Rad) was used to measure the absorbance at 570 nm.

15. General experimental setup for infected macrophage studies

RAW264.7 macrophages were seeded on coverslips in 24-well plates and incubated overnight at 37°C with 5% CO₂. The cells were used for bacteria invasion experiments when they reached 80% confluence. Afterward, the medium of each well was replaced with 1 mL of culture medium for RAW264.7 macrophages containing *S. aureus* at a multiplicity of infection (MOI) of 10. After infection, the cells were further processed depending on the specific experiment.

16. The subcellular localisation and cellular uptake of Mn-COF+L-Arg@Sr in infected macrophages

After infecting RAW264.7 macrophages with *S. aureus* (MOI of 10) for 1 hour, 100 μ L of culture medium containing RhB-labelled Mn-COF+L-Arg or RhB-labelled Mn-COF+L-Arg@Sr was added with a final concentration of 100 μ g·mL⁻¹. The macrophages were further incubated at 37 °C. At various expected time points, the culture medium was removed, and the macrophages were washed with PBS three times to remove the bacteria and nanoparticles outside the infected macrophages. Finally, samples were stained with DAPI (2 μ g·mL⁻¹) for 10 min and imaged by a confocal microscope (Nikon A1R).^{2, 3}

17. ROS detection in infected macrophages

After 1 h of infection, 200 μ L of culture medium containing RhB-labelled Mn-COF+L-Arg or RhBlabelled Mn-COF+L-Arg@Sr was added with a final concentration of 100 μ g·mL⁻¹. Control experiments were conducted in parallel without bacterial infection and without the addition of nanomaterials. The macrophages were further incubated at 37 °C. At the expected time point, the culture medium was removed, and the macrophages were washed with PBS three times to remove the bacteria and nanoparticles outside the infected macrophages. Then, DCFH-DA (10 μ M) was used for staining for 30min, during which US exposure (1 MHz, 1 W cm⁻², 50 % duty cycle, 5 min) was used, and DAPI (2 μ g·mL⁻¹) was used for staining for 10 min. Finally, samples were imaged by a confocal microscope (Nikon A1R) and analyzed by Image J software.

18. NO detection in infected macrophages

After 1 h of infection, the macrophages were further incubated at 37 °C. At the expected time point, the culture medium was removed, and the macrophages were washed with PBS three times to remove the bacteria and nanoparticles outside the infected macrophages. Finally, samples were stained with DAF-FM DA (10 μ M) for 20 min, during which US exposure (1 MHz, 1 W cm⁻², 50 % duty cycle, 5 min) was used, and DAPI (2 μ g·mL⁻¹) was used for staining for 10 min. Finally, samples were imaged by a confocal microscope (Nikon A1R) and analysed by Image J software.

19. ONOO- detection in infected macrophages

After 1 h of infection, the macrophages were further incubated at 37 °C. At the expected time point, the culture medium was removed, and the macrophages were washed with PBS three times to remove the bacteria and nanoparticles outside the infected macrophages. Finally, samples were stained with BBoxiProbe® O56 fluorescent dye (10 µM) for 30 min, during which US exposure (1 MHz, 1 W cm⁻², 50 % duty cycle, 5 min) was used, and DAPI (2 µg·mL⁻¹) was used for staining for 10 min. Finally, samples were imaged by a confocal microscope (Nikon A1R) and analysed by Image J software.

20. Studies on the colocalization of lysosomes by confocal fluorescence microscopy

After infecting RAW264.7 macrophages with *S. aureus* (MOI of 10) for 1 hour, 200 μ L of culture medium containing RhB-labelled Mn-COF+L-Arg@Sr was added with a final concentration of 100 μ g·mL⁻¹. The macrophages were further incubated at 37 °C. At the expected time point, the culture medium was removed, and the macrophages were washed with PBS three times to remove the bacteria and nanoparticles outside the infected macrophages. Finally, samples were stained with Lyso-Tracker Green fluorescent dye (1 μ M) for 30 min, and DAPI (2 μ g·mL⁻¹) was used for staining for 10 min. Finally, samples were imaged by a confocal microscope (Nikon A1R).

21. Elimination of the extracellular bacteria

For spread plate experiments, *S. aureus* was diluted with LB culture medium to 10⁶ CFU mL⁻¹. Typically, the bacteria solution was mixed with various nanoparticles in 24-well plates and incubated for 3 h. Control experiments were performed in parallel without the addition of nanoparticles. The final concentrations of nanoparticles in each group were 100 μg·mL⁻¹, respectively. After incubating with nanoparticles for 30 min, the bacterial suspensions in the groups of Mn-COF+US, Mn-COF+L-Arg+US, and Mn-COF+L-Arg@Sr+US with US treatment (1 MHz, 1 W cm⁻², 50 % duty cycle) were applied for 5 min. Finally, serial dilutions of the bacteria were made in PBS, and the number of surviving extracellular bacteria was determined by plating on LB agar plates.

22. Elimination of the intracellular bacteria in infected macrophages

To investigate the intracellular antibacterial activity of Mn-COF+L-Arg@Sr, RAW264.7 macrophages were seeded on coverslips in 6-well plates and incubated overnight at 37 °C with 5% CO2. The RAW264.7 macrophages were used for bacteria invasion experiments when they reached 80% confluence. Afterward, the medium of each well was replaced with 1 mL of culture medium for RAW264.7 macrophages containing S. aureus at a multiplicity of infection (MOI) of 10. After 1 h of infection, 1 ml of culture medium containing Mn-COF, Mn-COF+L-Arg, or Mn-COF+L-Arg@Sr was added with a final concentration of 100 μg·mL⁻¹. Control experiments were performed in parallel without the addition of nanoparticles. The macrophages were further incubated at 37 °C for another 2 h. Then, the infection mixture was removed and replaced with a culture medium for RAW264.7 macrophages supplemented with gentamycin (50 µg·ml⁻¹) to prevent the growth of extracellular bacteria. After incubation for 1 h, the infected macrophages in the groups of Mn-COF+US, Mn-COF+L-Arg+US, and Mn-COF+L-Arg@Sr+US were sonicated for 5 min (1 MHz, 1 W cm⁻², 50 % duty cycle). The survival of intracellular bacteria was assessed by using the plate colony counting method. Macrophages were lysed with PBS solution supplemented with 0.01 % Triton-X, and serial dilutions of the lysate were made in PBS. The number of the surviving intracellular bacteria was determined by plating on LB agar plates.

23. Bacterial membrane integrity assays

Mid-log-phase cells were diluted to an OD600 of 0.5 with HEPES buffer (5 mM HEPES, pH 7.4,

containing 20 mM glucose) and then treated with PBS, Triton X-100, Mn-COF, and Mn-COF+L-Arg@Sr with or without ultrasound and incubated in a 37 °C shaker for 30 min. After incubation, 200 μ L of cell suspension and 10 μ L of NPN probe (0.4 mM, dissolved in acetone) were mixed in a black polystyrene 96-well plate. The change of fluorescence intensity after 30 min was recorded on an enzyme label with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. PBS and 0.1 % Triton X-100 were employed as the negative and positive controls, respectively. All assays included three replicates and were repeated in three independent experiments.

24. Statistical analysis

Error bars represent the standard deviation from the mean (n = 3). Asterisks indicate statistically significant differences (*p < 0.05, **p < 0.01, ***p < 0.001).

Figures.



Figure S1. UV-Vis spectra of TAPP and Mn-TAPP.



Figure S2. (a) FT-IR spectra of Mn-COF, DHA, L-Arg, Mn-TAPP, and Mn-COF+L-Arg. (b) FT-IR spectra of DHA, TAPP, and B-COF. (c) FT-IR spectra of TAPP and Mn-TAPP.



Figure S3. LC-MS spectra of Mn-TAPP.



Figure S4. XRD patterns of B-COF and Mn-COF.



Figure S5. (a) XPS analysis of Mn-TAPP. (b) High-resolution Mn 2p XPS spectra of Mn-TAPP.



Figure S6. The typical TEM image of Mn-COF+L-Arg indicates that the adsorption of L-Arg on Mn-COF by electrostatic interaction does not destroy the Mn-COF structure.



Figure S7. (a) Mechanism of the Arg-to-NO conversion. (b) HPLC analysis indicates the retention time of PBS (4.45min), Arg (4.75min), and citrulline (4.97min). (c) Qualitative analysis of citrulline generation by HPLC during the conversion of Arg to NO by ROS.



Figure S8. Cytotoxicity of (a) Mn-COF+L-Arg and (b) Mn-COF+L-Arg@Sr towards 3T3 cells.



Figure S9. Cytotoxicity of (a) Mn-COF+L-Arg and (b) Mn-COF+L-Arg@Sr towards RAW264.7 macrophages.



Figure S10. Cellular uptake of Mn-COF+L-Arg@Sr by RAW264.7 macrophages infected with *S. aureus.* (a) Time-dependent fluorescence images of RhB-labeled Mn-COF+L-Arg in both uninfected (normal) and *S. aureus*-infected macrophages. (b) Time-dependent fluorescence images of RhB-labeled Mn-COF+L-Arg@Sr in both uninfected (normal) and *S. aureus*-infected macrophages. (c) Quantitative analysis of FI for RhB-labeled Mn-COF+L-Arg. (d) Quantitative

analysis of FI for RhB-labeled Mn-COF+L-Arg@Sr. (e) Quantitative analysis of FI for RhB-labeled Mn-COF+L-Arg, RhB-labeled Mn-COF+L-Arg@r, and RhB-labeled Mn-COF+L-Arg@Sr. All tested materials were used at a concentration of 100 μ g mL⁻¹. Error bars represent the standard deviation from the mean (n = 3). Asterisks indicate statistically significant differences (*p < 0.05, **p < 0.01, ***p < 0.001).

Discussion: The cellular uptake of Mn-COF+L-Arg by *S. aureus*-infected macrophages decreased by approximately 33% after 4 h of infection (Figs. S10a, c). This could be attributed to lysosomal destruction following infection, compromising cellular function. Similarly, the cellular uptake of Mn-COF+L-Arg@r by infected macrophages was diminished (Figs. S11, S12). Mn-COF+L-Arg@Sr demonstrated significantly higher cellular uptake efficacy in infected macrophages compared to Mn-COF+L-Arg due to bacteria-targeting properties of the Sr component (Figs. S10b, d). Indeed, significantly increased fluorescence intensity (FI) could be observed in *S. aureus*-infected macrophages treated with Mn-COF+L-Arg@Sr, which was also confirmed by flow cytometry and quantitatively analyzed by ICP-MS (Fig. S10e, Fig. S13a, Table S1).



Figure S11. Time-dependent cellular internalisation imaging of RhB-labelled Mn-COF+L-Arg@r by uninfected macrophages (normal macrophages) or *S. aureus*-infected macrophages.



Figure S12. Quantitative analysis of the fluorescence intensity from RhB-labelled Mn-COF+L-Arg@r.



Figure S13. Intracellular localization of Mn-COF+L-Arg@Sr in *S. aureus*-infected RAW264.7 macrophages. (a) Flow cytometry analysis of the cellular uptake of Mn-COF+L-Arg, Mn-COF+L-Arg@r, and Mn-COF+L-Arg@Sr by *S. aureus*-infected RAW264.7 macrophages. (b) Fluorescence imaging of bacteria treated with RhB-labelled Mn-COF+L-Arg, Mn-COF+L-Arg@r, and Mn-COF+L-Arg@Sr. (c) Bio-TEM images of *S. aureus*-infected RAW264.7 macrophages in various

experimental groups. (d) Co-localization fluorescence images of *S. aureus*-infected RAW264.7 macrophages with ROS, NO, and ONOO⁻ probes.

Discussion: Compared with Mn-COF+L-Arg and Mn-COF+L-Arg@r, the Mn-COF+L-Arg@Sr that had been internalized by infected host cells were attached to the intracellular *S. aureus* tightly. In other words, Mn-COF+L-Arg@Sr bound to extracellular *S. aureus* and been transported to intracellular *S. aureus*-located regions, guided by the attached *S. aureus*. These results could be further verified by fluorescence imaging (Fig. S13b). The colocalization of intracellular bacteria and lysosomes could be observed (Fig. S14). Fig. S13d demonstrated that Mn-COF+L-Arg@Sr treatment in *S. aureus*-infected macrophages under US irradiation induced substantial intracellular overproduction of ROS. The excess generation of NO and ONOO⁻ could be attributed to the reaction of ROS with L-Arg. Notably, these results also revealed the colocalization of green fluorescence from Mn-COF+L-Arg@Sr, demonstrating the dual bacterial and red fluorescence from Mn-COF+L-Arg@Sr, demonstrating the dual bacterial eradication. Fig. S15 indicated that US-mediated bacterial effect and NO-mediated distal bacterial eradication. Fig. S15 indicated that US-mediated NO release could induce bacterial membrane hyperpolarization, which increased membrane permeability and led to increased uptake of 1-N-phenylnaphthylamine.



Figure S14. The macrophages of *S. aureus* infection are colocalised with lysosomes.



Figure S15. NPN fluorescence intensity changes.

Table S1. Cell uptake of compounds calculated by ICP-MS.

| Sample | Mn ppm-1 | Mn ppm-2 | Mn ppm-3 | Average | Cell uptake |
|-----------------|----------|----------|----------|---------|-------------|
| Mn-COF+L-Arg | 22.14 | 22.13 | 22.09 | 22.12 | 19.23 wt% |
| Mn-COF+L-Arg@r | 35.85 | 34.53 | 34.64 | 35.01 | 30.44 wt% |
| Mn-COF+L-Arg@Sr | 44.88 | 44.70 | 45.48 | 45.02 | 39.14 wt% |

References.

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