Norharmane-Loaded Bacterial Cytoplasmic Membranes-Coated Nanoparticles Synergistically Enhance Polymyxin B Against *Pseudomonas aeruginosa* Infections by Disrupting Biofilms

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The PMφ coating has been shown to minimize immunogenicity in comparison with conventional bacterial outer membrane vesicles (BMOVs), while concurrently facilitating precise lung targeting in murine pneumonia models.

Materials and methods

In vitro bacteria targeting

The *P. aeruginosa* suspension (OD_{600} of 0.5) was incubated with 10 μg/mL FITC in 4℃ PBS for 2 h to gain FITC-labeled *P. aeruginosa* (*P. aeruginosa*-FITC) [1, 2]. *P. aeruginosa*-FITC was then co-incubated with NR-labeled NPs at a NR concertation of 200 ng/mL for 2 h and observed by a fluorescence microscope (EVOS M5000, ThermoFisher, USA). For flow cytometric analysis, *P. aeruginosa* (OD_{600} = 0.5) was co-incubated with different formulations at the same NR concentration (200 ng/mL) for 2 h and analyzed by flow cytometer (Cytoflex S, Beckman, USA).

In vitro biofilm penetration

The *P. aeruginosa* suspension (OD_{600} = 0.5) was serially diluted 1000-fold with LB medium. Subsequently, 1 mL of the diluted suspension was added to 35-mm confocal dishes and incubated for 24 h at 37 ℃. Thereafter, the dishes were washed three times with PBS to remove planktonic bacteria. Then, 1 mL of fresh LB medium containing NR-labeled NPs (200 ng/mL NR) was added to each well and incubated for an additional 2 h. Following this incubation period, the wells were gently washed with cold PBS thrice. Then, the wells were fixed with 4% paraformaldehyde for 15 min at 25 ℃ and scanned by a laser scanning confocal microscope (STELLARIS, Leica, Germany).

MIC determination

The *P. aeruginosa* suspension (OD_{600} of 0.5) was diluted 1000-fold with LB medium. 0.1 mL of the diluted suspension was added to each well of a 96-well plate. Subsequently, an additional 0.1 mL of LB medium containing a gradient concentration of NOR or PMB was added. The plate was subjected to an incubation process that involved a constant rotation speed of 100 rpm/min at a temperature of 37 ℃ for a duration of 16 h. The OD_{600} value of each well was measured using a microplate reader (Synergy H1, Biotek, USA). The MIC was determined as the drug concentration at which the bacterial inhibition rate exceeds 90%.

In vitro biofilm formation inhibition

The *P. aeruginosa* suspension (OD_{600} = 0.5) was serially diluted 1000-fold with fresh LB medium. 0.1 mL of the diluted suspension was added to each well of a 96-well plate. Subsequently, an additional 0.1 mL of LB medium containing NPs (10 or 20 μg/mL NOR) and PMB (20 μg/mL) was added. The plate was subjected to a 24-h culture at 37°C in a static state. Following the incubation, the wells were washed with PBS three times. Then, 0.1 mL of 0.1% CV was added to each well and allowed to sit for 15 min. Following a third round of washes with PBS, the purple precipitate in each well was dissolved with 0.1 mL of 33% acetic acid. The OD_{570} value of each well was measured by means of a microplate reader.

Furthermore, the number of residual bacteria in the biofilm was also investigated [3]. The biofilm was dispersed in 100 μL PBS using a sonicating water bath. Serial dilutions (10^2-10^8) were prepared, and 10 μL of each dilution was spread on LB plates for counting.

To intuitionistic observation of the destruction of biofilm after antibiotic incubation, a holographic 3D microscope, Nanolive (3D Cell Explorer, Nanolive SA, Switzerland), was used to scan the

residual biofilm through the z-axis [3]. The software ‘‘Steve 1.6.3496’’ (Nanolive SA, Switzerland) was used for image acquisition and reconstruction.

In vivo pharmacokinetics

A total of 24 ICR mice (6 weeks old, 17-19 g) were divided into two groups and intraperitoneally injected with NOR or PMφ-PLGA-NOR at a NOR dosage of 5 mg/kg, respectively. At predetermined time points, 0.25 mL of blood was collected from the vein of the fundus plexus and placed in heparin-anticoagulated centrifuge tubes. The tubes were then subjected to centrifugation at 5000 g for 15 min at 4°C. A volume of 100 μL of plasma was combined with 10 μL of IS standard solution and 300 μL of acetonitrile. Following the vortex mixing stage, the mixture underwent a centrifugation process at 12,000 g for a duration of 10 min at 4 °C. The supernatant was extracted and maintained at 37 °C for the purpose of nitrogen blowing. Following the drying process, the samples were redissolved in 100 μL of the initial mobile phase (a 10% methanol aqueous solution). The samples were subjected to centrifugation at 12,000 g at 4 °C for 10 min. The supernatant was collected for liquid chromatograph-mass spectrometer (LC-MS) analysis. The chromatographic conditions were as follows:

The Poroshell 120 SB-C18 chromatographic column (2.1×50 mm, 1.9 μm, Agilent, USA) was selected as the chromatographic column. The mobile phase was configured as a 10% methanol solution in water for the initial 5 min, followed by a transition to a 10-100% methanol solution in water for the subsequent 5-20 min. This was then followed by a return to a 10% methanol solution in water for the final 20-25 min. The flow rate was set at 0.3 mL/min, and the injection volume was set at 1 μL.

A tandem quadrupole mass spectrometer (TRIPLE QUAD 3500, AB Sciex, USA) was used. Ion source: electrospray ion source, positive ion mode; Scanning mode: multiple reaction monitoring; Air curtain air: 35 Psi. Collision gas, 8 Psi. Spray voltage: 5500 V; Atomization temperature: 550 ℃; Aerosol: 55 Psi; Auxiliary gas: 55 Psi; To cluster voltage: 130 V; Into the voltage: 10 V; Collision chamber injection voltage: 6 V.

In vitro and in vivo biosafety evaluation

Mouse fibroblast L929 cells were seeded in a 96-well plate at a density of 5,000 cells per well. Following a 12 h incubation period, the existing medium was removed and 100 μL of fresh medium containing NOR or Mφ-PLGA-NOR was added. Following a 24 h incubation period, 10 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h. Thereafter, the medium was removed and 100 μL of DMSO was added to each well to dissolve the purple crystal. The OD_{570} value of each well was subsequently measured by means of a microplate reader.

For the hemolysis experiment, 2 mL of blood was collected from the abdominal aorta of ICR mice and diluted with 30 mL of ice-cold PBS. The blood was subjected to centrifugation at 3,000 rpm for 20 min to collect erythrocytes. The erythrocytes were then incubated with NPs at 37 °C for 4 h and then subjected to centrifugation at 3000 rpm for 20 min. The supernatant was collected, and the OD_{540} value was measured.

For *in vivo* biosafety test, healthy ICR (6 weeks old, 17-19 g) mice were randomly divided into four groups (PBS, PMB, PMB+NOR, and PMB+PMφ-PLGA-NOR) (n=3). The mice were administered intraperitoneally with various formulations (NOR 10 mg/kg, PMB 3 mg/kg) every 12 h for a total of two times. 24 h after the last administration, the mice were euthanized, and their hearts, spleens, kidneys, livers, and lungs were collected and fixed in 4% paraformaldehyde solution. H&E staining of the sections was carried out.

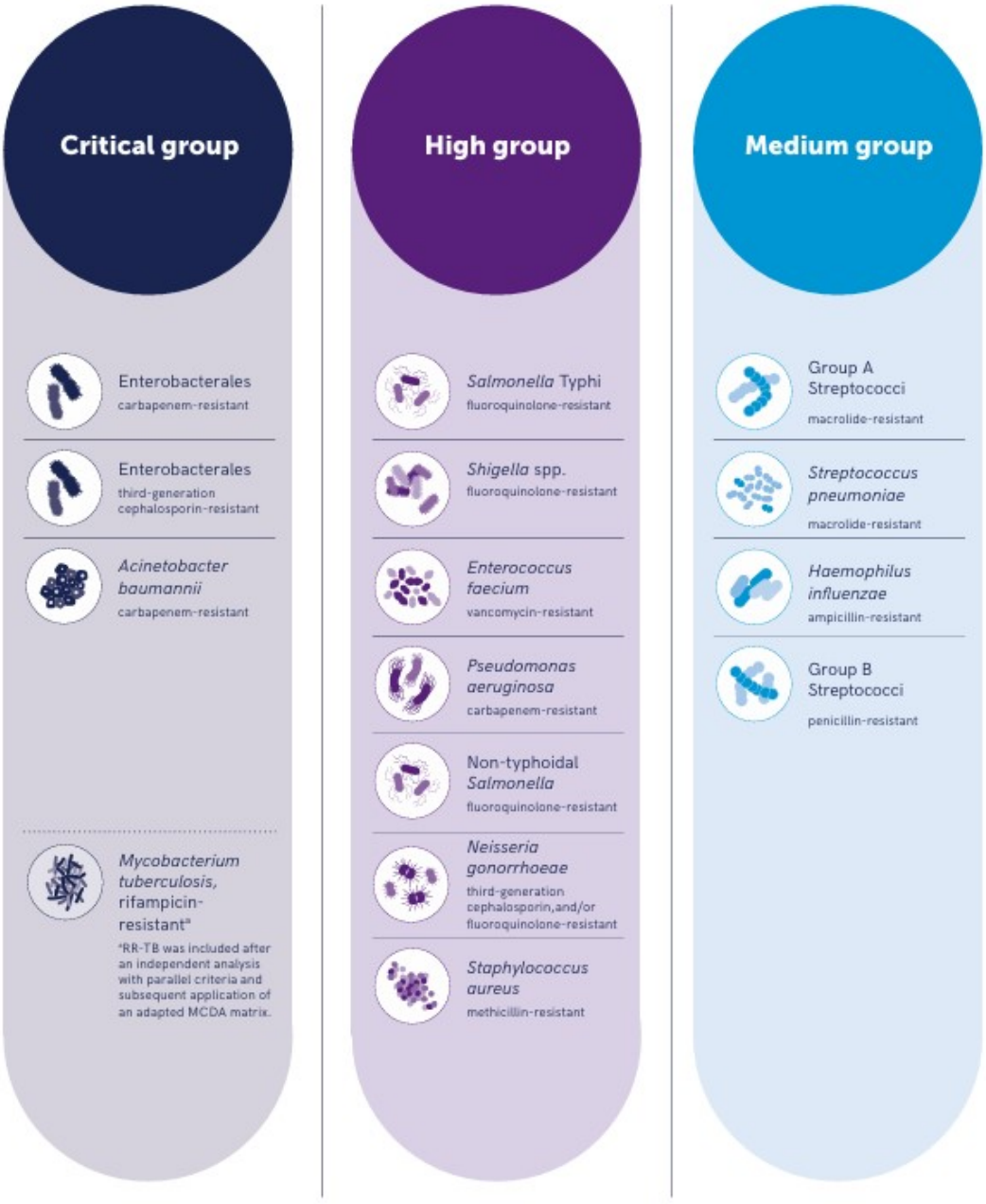

Figure. S1 WHO Bacterial Priority Pathogens List, 2024.

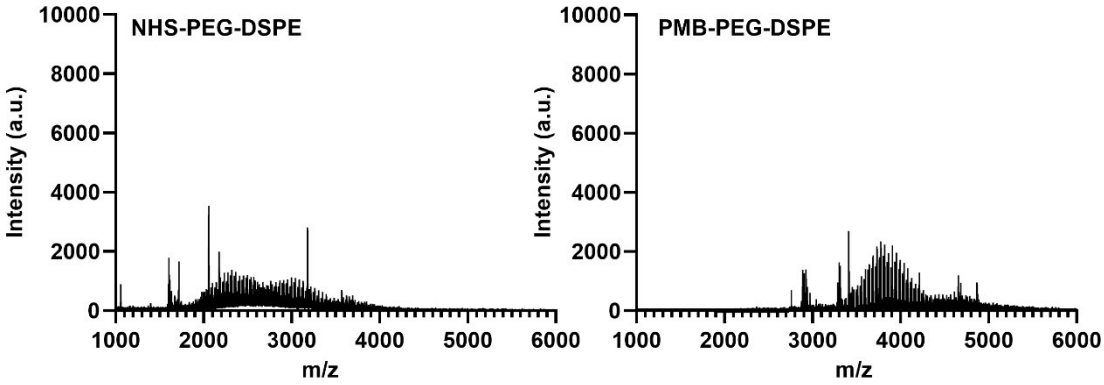

Figure. S2 The MALDI-TOF-MS spectrum of NHS-PEG-DSPE and PMB-PEG-DSPE.

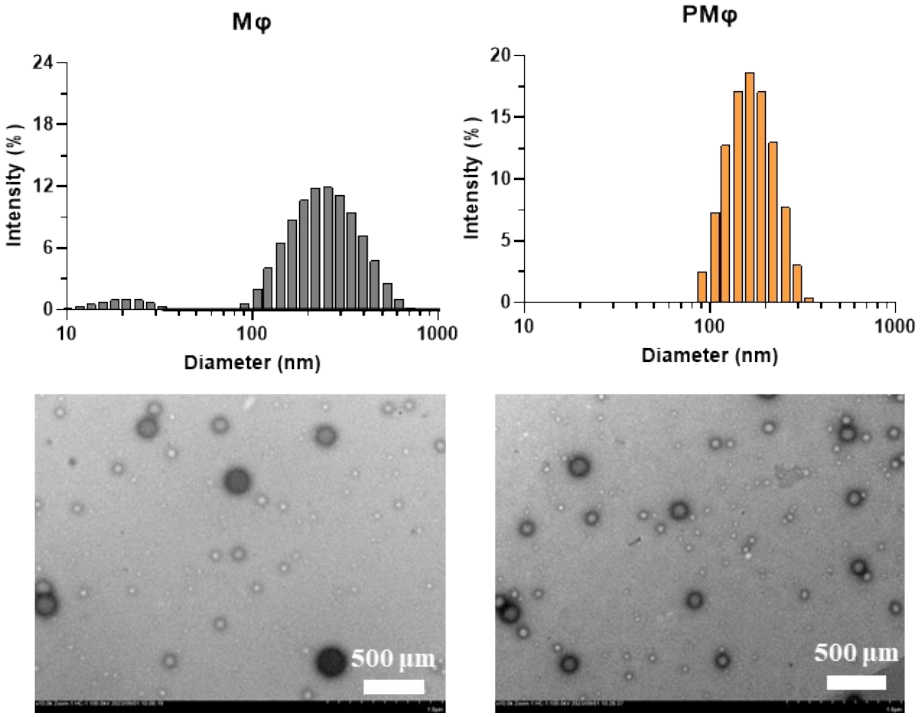

Figure S3. Size distribution and TEM images of Mφ and PMφ.

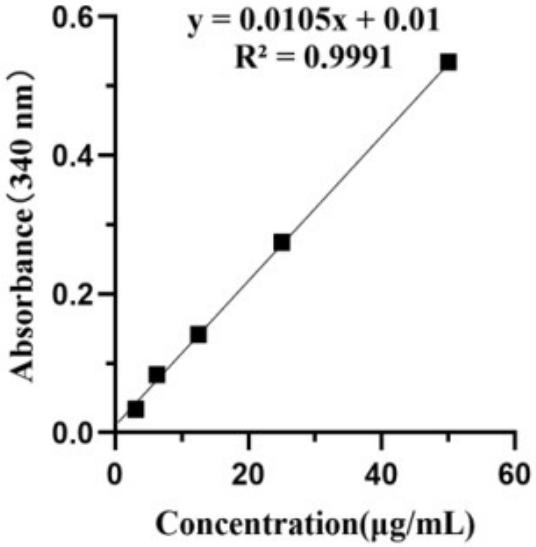

Figure S4. Quantitative curve for determining NOR using the microplate spectrophotometer method. For drug-loading quantification, where the composition is relatively simple with a relatively high NOR content, the NOR content can be measured using a microplate spectrophotometer.

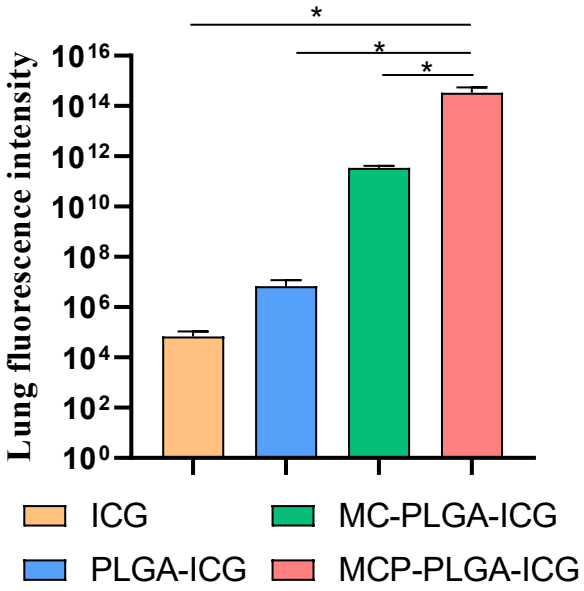

Figure S5. Quantitative analysis of fluorescence in the lungs of mice after administration of different ICG-labeled preparations (n=3). Data are expressed as mean ± SD.

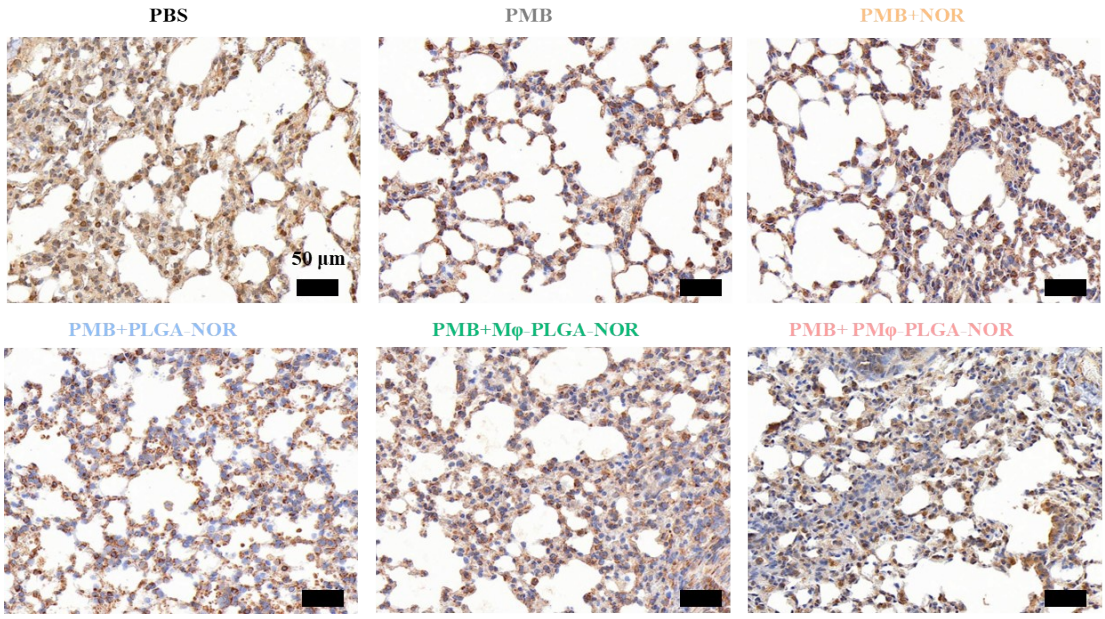

Figure S6. Immunohistochemical sections of TNF-α were further provided in the lung tissues of different treated mice.

Reference

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[3] Y. Cai, Y.T. Chu, Y.B. Gong, Y.L. Hong, F.H. Song, H. Wang, H.W. Zhang, X.R. Sun, Enhanced Transdermal Peptide-Modified Flexible Liposomes for Efficient Percutaneous Delivery of Chrysomycin A to Treat Subcutaneous Melanoma and Intradermal MRSA Infection, Advanced Healthcare Materials 12(26) (2023). 10.1002/adhm.202300881.