Supplementary information for

LETTER TO THE EDITOR

Inhaled heparin polysaccharide nanodecoy against SARS-CoV-2 and variants

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Materials and methods

1.1. Materials

Heparin sodium (average molecular weight: 15,000–19,000, termed HMWH),

dalteparin sodium (average molecular weight: 5600–6400, termed LMWH), DiR iodide, DiI perchlorate, and Firefly Luciferase Reporter Assay Kit were purchased from Melone Pharmaceutical Co., Ltd. (Dalian, China). Chitosan (average molecular weight: 25,000) was obtained from Yunzhou Biochemistry Co., Ltd. (Qingdao, China). Heparinase I and III blends were obtained from Sigma–Aldrich (St. Louis, MO, USA). Other reagents were provided from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

1.2. Cell lines, pseudovirus, and animals

The 293T cells were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in Dulbecco's modified Eagle's medium (DMEM). The 293T-hACE2 cells were constructed by stable transfection of hACE2 using Lentiviral HBLV-h-Ace2-3xflag-ZsGreen-PURO (Hanbio Biotechnology Co., Ltd., Shanghai, China). SARS-CoV-2 S pseudoviruses, including wide-type, Delta (Lineage B.1.617.2 Lineage), and Delta plus (B.1.617.2.1/AY.1), were purchased from Ji Manchu Biotech Co., Ltd. (Shanghai, China). The BALB/c female mice aged 6–8 weeks were purchased from Shanghai Laboratory Animal Center (SLAC) Co., Ltd. (Shanghai, China) and raised under pathogen-free conditions. All animal experiment procedures followed the institutional ethical guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

1.3. Preparation and characterization of heparin NPs

The heparin NPs were prepared by the electrostatic interaction *via* chitosan crosslinking. The appropriate amount of chitosan was added to 1% acetic acid solution for dissolving under the heat condition, and the final concentration of chitosan was adjusted to 1 mg/mL by adding ultrapure water. Meanwhile, the heparin solution (2 mg/mL) was prepared using ultrapure water. The solutions of chitosan and heparin were directly mixed at an equal volume and then stirred overnight at a speed of 1000 rpm by using a magnetic stirrer (X85-2S, Meiyinpu, Shanghai, China) to obtain the heparin NPs.

The particle size and ζ-potential of heparin NPs were detected by the dynamic light

scattering technique (Zetasizer Nano-90ZS, Malvern, UK). The morphology of the heparin NPs was observed using a cryo-transmission electron microscope (cryo-TEM, FEI Talos Arctica G2, Thermo Fisher, Waltham, USA) at 200 kV. The stability of the heparin NPs in PBS (pH 7.4) was evaluated by monitoring the particle size change using the dynamic light scattering technique.

1.4. Binding of heparin NPs and SARS-CoV-2 pseudoviruses

An appropriate amount of pseudoviruses was added to the heparin NP suspension, and they were mixed evenly with a pipette and then incubated for 2 h. After dilution to an appropriate concentration using PBS, the particle size was measured. The morphology of the complex of the heparin NPs and pseudoviruses was observed using cryo-TEM (200 kV).

1.5. Inhibition of pseudovirus infection in the host cells

The cells were inoculated in 96-well plates at a density of 5000 cells per well. After 12 h, the pseudovirus (1.85×10^5 TU/mL, 0.1 mL per well), heparin or the heparin NPs were added to the cells, respectively, and then cultured for 6 h. Afterward, the medium was discarded, with replacement with fresh culture medium, and the cells were cultured for 48 h. The luciferase expression in the wells was detected by a standard protocol with Firefly Luciferase Reporter Assay Kit (Meilunbio, Dalian, China). The luciferase activity of each well was further normalized to the protein concentration measured by a BCA Microplate Protein Assay Kit (Beyotime, Shanghai, China).

1.6. Cell surface-heparan elimination by heparinase and its effect on transfection

The cells were inoculated in 96-well plates at a density of 5000 cells per well. After 12 h, the culture medium was replaced by the heparinase solution with a concentration of 10 mU/mL. The cells were then incubated for 30 min. After incubation, the heparinase solution was substituted for the pseudovirus suspension for 6 h. Afterward, the medium was discarded, with replacement with fresh culture medium, and the cells were cultured for 48 h. The luciferase expression was detected as mentioned above.

1.7. Preparation of DiR or DiI-labeled pseudoviruses

The membrane dye (DiR or DiI, 5 mg/mL) was added to the pseudovirus suspension

with a volume ration of 1:7 and incubated at 37 ℃ for 30 min. The free dye was removed by using a centrifugal ultrafiltration tube (MWCO: 10,000) at a speed of 4000 rpm (Heraeus Multifuge X1R, Thermo Fisher,Germany) for 30 min.

1.8. Preparation of bone marrow-derived macrophages (BMDMs)

The BALB/c female mice were humanely sacrificed and the bone marrow cells were collected by using a standard method as previously reported[29]. In brief, the bone marrow cells were rinsed with serum-free DMEM and then cultured in the complete DMEM containing 20 ng/mL M-CSF (Peprotech, Rocky Hill, USA) for 4 days to induce the differentiation of BMDMs. Afterward, the culture medium was replaced by another conditioned medium containing lipopolysaccharide (LPS, 100 ng/mL, Sigma–Aldrich, St. Louis, USA) and IFN-γ (20 ng/mL, Peprotech) for 24 h culture to induce the M1-type macrophages.

1.9. Intracellular uptake of the pseudovirus

The cells (293T or 293T-hACE2) were inoculated in 96-well plates at a density of 5000 cells per well. After 12 h, the DiI-labeled pseudovirus, or the mixture of the DiI-labeled pseudovirus and the heparin NPs were added to the cells, respectively, for 2 h incubation. The uptake efficiency of the pseudoviruses was analyzed by fluorescence microscopy and flow cytometry.

The DiI-labeled pseudovirus, or the mixture of the DiI-labeled pseudovirus and the heparin NPs were also added to the macrophages mentioned above for 2 h incubation. The clearance efficiency of the virus/NPs complex by the macrophages was investigated by determining the fluorescence signal by the fluorescence microscope and flow cytometry.

1.10. Inhibition of pseudovirus infection to the lung in vivo

Pseudovirus infection in the lungs and different treatments for infected mice were performed through a micro liquid intratracheal atomizer (HY-LWH03) and small animal laryngoscope (HY-SHJOI, YSKD Bio-Technology, Beijing, China). Briefly, the micro liquid intratracheal atomizer was extended to the mouse trachea with the aid of the small animal laryngoscope and to deliver the atomized liquid into the mouse's lung.

The BALB/c female mice were given the same amount of DiR-labeled pseudovirus by intratracheal administration, and the mice were then randomly divided into three groups. After 30 min, the three groups were given an equal volume (20 μL) of PBS, heparin solution (2 μmol/L), and the heparin NPs (2 μmol/L), respectively, *via* pulmonary administration. After 24 h, the mice were euthanized and the lung tissues were collected for *ex vivo* imaging by the IVIS imaging system (Caliper PerkinElmer, Hopkinton, USA).

Additionally, the quantification of the luciferase expression in the lung was determined by quantitative real-time polymerase chain reaction PCR (qRT-PCR). Total RNA was isolated from the lung with Trizol (Tiangen, Beijing, China). The reverse transcription was completed with the iScriptTM gDNA Clear cDNA Synthesis Kit (BioRad, Hercules, USA). The multiple kits (SYBR Premix Ex TaqTM, RR036A, Takara Bio, Kusatsu, Japan) were used for the real-time PCR, which was finished in an ABI 7500FAST Sequence Detector System (ABI, Foster City, USA). Actin was used as a loading control and its forward and reverse primers were respectively 5′-GGTCATCACTATTGGCAACG-3′ and 5′-ACGGATGTCAACGTCACACT-3′. The luciferase forward and reverse primers were 5′-AATGTCCGTTCGGTTGGCAG-3′ and 5′-GGCTGCGAAATGCCCATACT-3′.

1.11. Data analysis

All data were analyzed by in the form of mean ± standard deviation (SD). *T*-test, one-way ANOVA, and two-way ANOVA were used for statistical analysis. $P<0.05$ were considered as statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$), ns represents no significance.

Supplementary figures

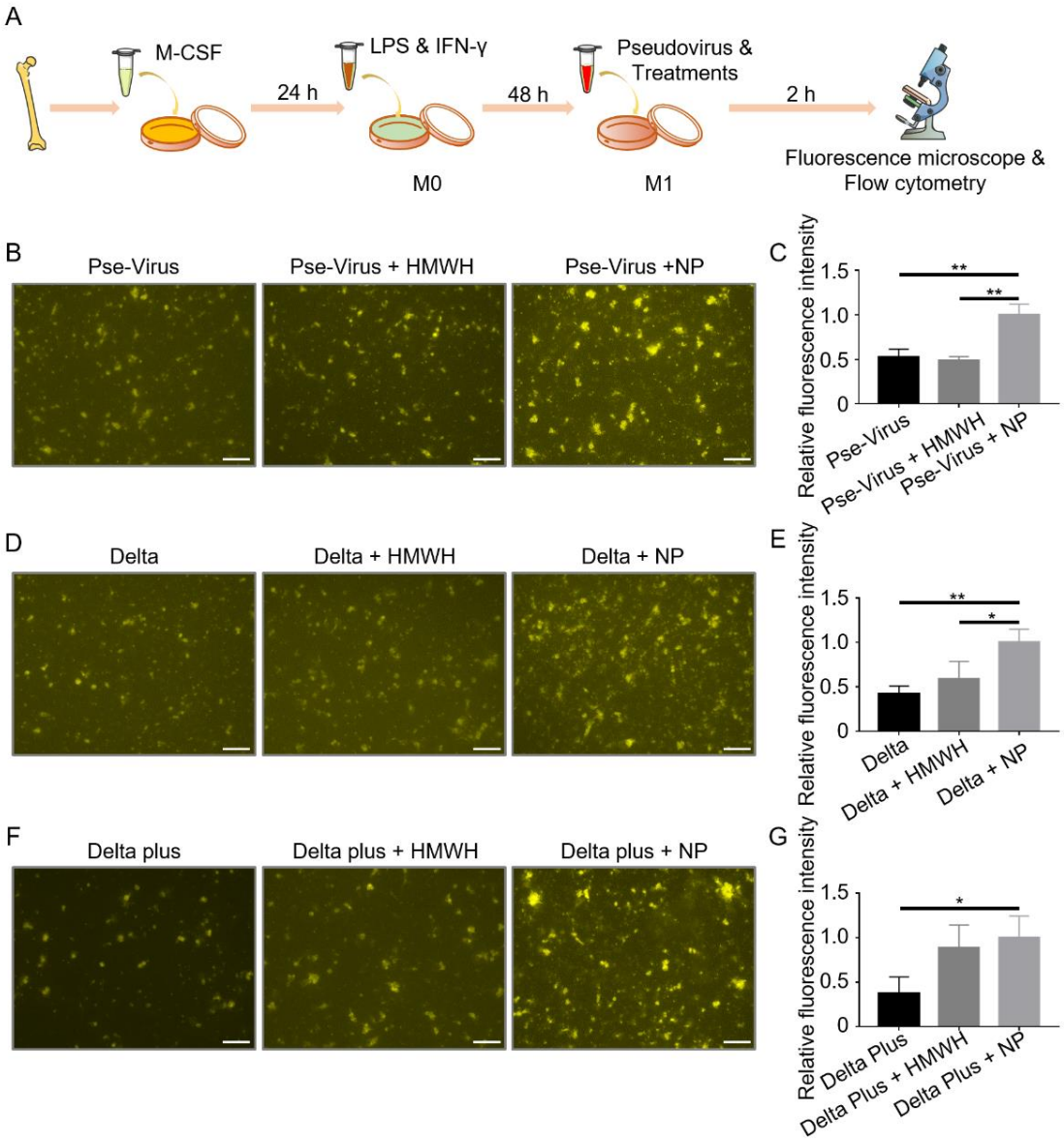

Figure S1 The phagocytosis efficiency of the heparin NP/virus in macrophages. (A) Schematic illustrating the clearance of the NP/virus by macrophages. Fluorescence images and flow cytometry assay of the phagocytosis of SARS-CoV-2 pseudovirus (B and C), Delta pseudovirus (D and E), and Delta plus pseudovirus (F and G) in macrophages after treatments. Scale bar=100 μm. Data are presented as mean ± SD (*n* = 3); **P*<0.05, ***P*<0.01.