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Supporting Information

Inflammation-Responsive Hierarchical Delivery of Anti-Inflammatory siRNA and Peptide Alleviates Cytokine Storm in Pneumonia

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SUPPLEMENTARY METHODS

1. Materials, cells, and animals

All chemicals and solvents were purchased from Aladdin (Shanghai, China) or Energy Chemical (Shanghai, China) unless otherwise indicated. RBP (99%) was synthesized by Top-Peptide Biotechnology (Nanjing, China), and its amino acid sequence was KLKEKYEKDIAAYRAKGKPDAAKKGVVKAEKSKKKKEC. siTNF-α, ^{Cy3}siTNF-α, ^{FAM}siTNF-α, siNC, and primers were purchased from GenePharma (Shanghai, China), and their sequences were listed in Table S2 and S3. LPS (from *Escherichia coli 0111:B4*) and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All ELISA kits were purchased from Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). Myeloperoxidase assay kit was purchased from eBioscience (San Diego, CA, USA). All antibodies were purchased from Abcam (Shanghai, China).

RAW 264.7 (mouse monocyte macrophage) and Calu-3 (human lung adenocarcinoma) cells were purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

Male Balb/c mice (6-8 weeks) were purchased from Shanghai Slaccas Experimental Animal Co., Ltd. (Shanghai, China), and were housed in a specific pathogen-free room, four to a cage, with access to water ad libitum, a 12:12 h light-dark cycle (7:00 am - 7:00 pm), and a temperature of 25 ± 1 °C. The animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee, Soochow University. The approval number for the laboratory is SYXK(Su)2017-0043.

2. Synthesis of DPP

Firstly, *γ*-(4-propargyloxybenzyl)-L-glutamic acid-based *N*-carboxyanhydride (POBLG-NCA) was synthesized according to reported procedures (yield 83%),[1,2] and its chemical structure was confirmed by ^{1}H nuclear magnetic resonance (^{1}H NMR). Then, poly(γ-(4-propargyloxybenzyl)-L-glutamate (PPOBLG) was synthesized *via* ring opening polymerization (ROP) of POBLG-NCA as initiated by generation 3 poly(amidoamine) dendrimer (G3-PAMAM). Briefly, POBLG-NCA (0.86 g, 2.71 mmol) was dissolved in anhydrous *N*,*N*-dimethylformamide (DMF, 5 mL) in a vial under nitrogen, into which a solution of G3-PAMAM in DMF (0.1 mol/L,

8.5 μL, 0.85 μmol) was added with a syringe. The reaction mixture was stirred at RT for 72 h. The product PPOBLG was precipitated with distilled water (60 mL) and collected as white solid (0.70 g, yield 93%). The chemical structure of PPOBLG was confirmed by ^{1}H NMR, and the molecular weights (MWs) were determined by gel permeation chromatography (GPC).

Then, PPOBLG (30 mg, 0.08 mmol of alkynyl group) and 6-azidohexyl guanidine (40 mg, 0.22 mmol) were dissolved in DMF (3 mL) in a glass vial (10 mL) in the glovebox, followed by the addition of *N*,*N*,*N'*,*N''*,*N''*-pentamethyldiethylenetriamine (PMDETA, 34 μL, 0.125 mmol) and CuBr (18 mg, 0.125 mmol). The mixture was stirred at RT in darkness for 24 h and quenched by exposure to air. HCl (1 mol/L, 3-4 mL) was then added until the solution became colorless. The product was dialyzed against deionized water (MWCO = 3.5 kDa) for 3 d and lyophilized to obtain DPP as white solid (yield 86%). The structure of DPP was characterized by ^{1}H NMR. The secondary structure of DPP was determined by circular dichroism (CD).

3. Synthesis of PLL

Firstly, *N*(ε)-benzyloxycarbonyl-L-lysine (ZLL, 5.0 g, 17.85 mmol) and triphosgene (3.50 g, 11.90 mmol) were dissolved in THF (100 mL) and reacted in 250-mL round-bottom flask at 50 °C for 1.5 h. The crude product was obtained after removal of THF under vacuum and purified by recrystallization in ethyl acetate/n-hexane (v/v = 1/10) at -20 °C for three times. The product *N*(ε)-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride (ZLL-NCA) was obtained as white powder (4.61 g, yield 85%). The chemical structure of ZLL-NCA was confirmed by ^{1}H NMR. Then, ZLL-NCA (1.70 g, 5.55 mmol) was dissolved in DMF (25 mL), into which *n*-butylamine (0.5 M in DMF, 555 μL, [M]/[I] = 100) was added. The mixture was stirred at RT for 3 d in a glovebox. Poly(*N*(ε)-benzyloxycarbonyl-L-lysine) (PZLL) as the product was obtained *via* precipitation in anhydrous ether followed by filtration (1.9 g, yield 94%). The chemical structure of PZLL was confirmed by ^{1}H NMR, and the MWs were determined by GPC.

PLL was obtained *via* hydrolysis of PZLL. Briefly, PZLL (2.50 g) was dissolved in trifluoroacetic acid (TFA, 25 mL), and then hydrogen bromide/acetic acid solution (HBr/HAc, w/w = 1/3, 8 mL) was slowly added under ice bath. After reaction for 1 h at RT, the solution was precipitated into diethyl ether. The crude product was then dialyzed (MWCO = 3500 Da) against deionized water for 3 d, and then lyophilized to obtain PLL (1.20 g, yield 90%). The chemical structure of PLL was confirmed by ^{1}H NMR.

4. Characterization of NCs

The particle sizes and zeta potentials of NCs were measured by Zetasizer (Nano-ZS, Malvern). To evaluate siTNF-α condensation level, freshly prepared NCs were subjected to electrophoresis on 2% agarose gel at 90 V for 20 min. The stability of NCs was evaluated by measuring the particle size after incubation in PBS or mucin solution (0.3% in saline, w/v) for 48 h. To evaluate the siRNA release, RDDsT NCs containing ^{Cy3}siTNF-α were incubated in PBS or mucin solution. At predetermined time points, the solution was centrifuged at 20,000 g and 4 °C for 10 min, and the ^{Cy3}siTNF-α content in the supernatant was determined by spectrofluorimetry ($\lambda_{ex}/\lambda_{em}$ = 550/565 nm).

5. Evaluation of the pH sensitivity of RDDsT NCs

RDDsT NCs (RBP/PD/DPP/siTNF-α = 40/10/10/1, w/w/w/w) were prepared as described above. Sizes and zeta potentials of NCs were measured after incubation in deionized water at pH 6.8 or 7.4 for 30 min. The non-responsive RSDsT NCs were used as the control. The FRET analysis was further employed to investigate the acid-triggered PD detachment from RDDsT NCs. Briefly, RDDsT NCs comprised of ^{Cy3}siTNF-α and ^{Cy5}PD as the FRET pair were incubated in deionized water at pH 6.8 or 7.4 for 30 min. The fluorescence emission spectrum was recorded within the rage of 560-750 nm (λ_{ex} = 550 nm). The non-responsive RSDsT NCs comprised of ^{Cy3}siTNF-α and ^{Cy5}PS were used as the control.

6. Cellular uptake and intracellular kinetics of NCs

RAW 264.7 cells were seeded on 96-well plates at 1×10^4 cells/well and cultured for 24 h. Cells were incubated with various ^{FAM}siTNF-α-containing NCs (DsT, DDsT, SDsT, RDDsT, or RSDsT) at 1 μg ^{FAM}siTNF-α/mL in opti-MEM (pH 6.8 or 7.4) for 4 h. Cells were then treated with trypan blue solution (0.4%) for 10 min, washed with cold PBS containing heparin (20 U/mL) for three times, and lysed with the RIPA lysis buffer (100 μL/well). ^{FAM}siTNF-α content in the lysate was monitored by spectrofluorimetry ($\lambda_{ex}/\lambda_{em}$ = 492/518 nm) and protein level was quantified using the BCA protein assay kit. Uptake level was expressed as μg ^{FAM}siTNF-α per mg protein.

To visualize the cellular internalization of NCs, RAW 264.7 cells were seeded on a glass-bottomed dish ($\Phi = 20$ mm) at 4×10^4 cells/dish and cultured for 24 h. Cells were incubated with RDDsT NCs or RSDsT NCs containing ^{FAM}siTNF-α at 1 μg ^{FAM}siTNF-α/mL in opti-MEM (pH 6.8 or 7.4) for 4 h. Cells were then treated with trypan blue solution (0.4%) for 10 min, washed with cold PBS containing heparin (20 U/mL) for three times, and observed by CLSM (Leica, TCS SP5, Germany).

To observe the endolysosomal escape of NCs, RAW 264.7 cells seeded on a glass-bottomed dish were incubated with ^{FAM}siTNF-α-containing RDDsT NCs at 1 μg ^{FAM}siTNF-α/mL in opti-MEM (pH 6.8) for 2 h or 4 h. Cells were then treated with trypan blue solution (0.4%) for 10 min, washed with cold PBS containing heparin (20 U/mL) for three times, stained with Hoechst 33258 (5 μg/mL) for 10 min and LysoTracker Red (200 nM) for 1 h, and observed by CLSM. Pearson's correlation coefficient between ^{FAM}siTNF-α and LysoTracker Red-stained endolysosomes were calculated by the ImageJ software.[3]

7. *In vitro* gene silencing in macrophages

RAW 264.7 cells seeded on 96-well plates were incubated with PBS, RBP, or various NCs (PEI 25k/siTNF-α (5/1, w/w, abbreviated as PsT), DsT, DDsT, RDDsT, and RDDsC) in opti-MEM (pH 6.8) for 4 h at 1 μg siTNF-α/mL and 40 μg RBP/mL. The medium was then replaced by fresh medium containing 10% FBS, and cells were further cultured for 20 h before challenge with LPS (7.5 ng/mL) for 5 h. The TNF-α concentration in the medium was determined using an ELISA kit, and the result was expressed as percentage TNF-α concentration of control cells that received PBS treatment. In addition, cells were collected, and the TNF-α mRNA level was determined by real-time polymerase chain reaction (PCR, Bio-Rad CFX connect).

To further evaluate the pH-dependent gene-silencing efficiency, RAW 264.7 cells seeded on 96-well plates were incubated with RDDsT NCs or RDDsC NCs at 1 μg siRNA/mL in opti-MEM (pH 6.8 or 7.4) for 4 h. Cells were then challenged with LPS and incubated as described above. The TNF-α concentration in the medium was determined by an ELISA kit and the TNF-α mRNA level in cells was determined by real-time PCR.

8. MPT in CF mucus

CF sputum from patients was diluted with PBS for 20 folds. ^{Cy3}siTNF-α-containing NCs (DsT, DDsT, and RDDsT) were mixed with CF sputum (300 μL) at 10 μg ^{Cy3}siTNF-α/mL and transferred to custom-made microwells (400 μL) after 30-min equilibrium at 37 °C. 20-s movies at 66.7 ms temporal resolution were acquired by an Evolve 512 EMCCD camera (Photometrics, Tucson, AZ) equipped on an inverted epifluorescence microscope (Observer Z1, Zeiss; Thornwood, NY) with a 100 × 1.4 NA objective. Imaris software was used for the analysis of Brownian movement, and the track of individual NCs was expressed in the form of <MSD> as a function of time scale (τ). Moreover, the distributions of D_{eff} were also counted.[4]

9. *In vitro* permeation across Calu-3 cell monolayers

AIC of Calu-3 cells, a well-established *in vitro* model of bronchial epithelia with secreted mucus layer,[5] was adopted to evaluate the trans-mucus/trans-epithelia penetration of NCs. Briefly, Calu-3 cells were seeded on 24-well Transwell inserts (Corning, NY, USA) at 5 × 10^4/well and put into the 24-well culture plates. The trans-endothelial electrical resistance (TEER) value was daily measured during days 7 to 14. The medium in the apical compartment was removed on day 4 while the medium in the basolateral side was refreshed every day. When the TEER value reached 700 Ω·cm^2 (usually within 14 d), the cell monolayers were used for the permeation studies. Hank's balanced salt solution (HBSS, 500 μL) containing 1% bovine serum albumin (BSA) was added to the basolateral side, and various ^{Cy3}siTNF-α-containing NCs (DsT, DDsT, and RDDsT, 30 μg ^{Cy3}siTNF-α/mL in HBSS containing 1% BSA, 200 μL) were added to the apical side. After incubation at 37 °C for 6 h, the medium in the basolateral side was harvested, and the amount of ^{Cy3}siTNF-α was determined by spectrofluorimetry ($\lambda_{ex}/\lambda_{em}$ = 550/565 nm). The penetration capabilities of the NCs were represented by the P_{app} using the equation of $P_{app} = Q/Act$, where Q is the amount of permeated ^{Cy3}siTNF-α (ng), A is the diffusion area of the cell monolayers (cm^2), c is the initial concentration of ^{Cy3}siTNF-α at the apical side (ng/cm^3), and t is the transport time (s).

10. *In vivo* mucus penetration and pulmonary distribution

At 2 h post LPS challenge, ALI mice were intratracheally injected with DsT NCs or RDDsT NCs containing ^{Cy3}siTNF-α at 150 μg ^{Cy3}siTNF-α/kg. One hour later, animals were sacrificed and lung tissues were collected, washed with PBS, embedded in optimal cutting temperature

compound (OCT), cryo-sectioned at 10-μm thickness, stained with DAPI for 10 min, and observed by CLSM. The fluorescence intensity of ^{Cy3}siTNF-α in lung tissues was quantified by ImageJ software.

11. Biodistribution of NCs

ALI mice were intratracheally injected with RDDsT NCs containing ^{Cy3}siTNF-α as described above. At predetermined time points, animals were sacrificed and major organs (heart, liver, spleen, lung, and kidney) were collected, washed with PBS, and homogenized with 1% Triton X-100. After centrifugation (3,000 g, 4 °C, 10 min), ^{Cy3}siTNF-α content in the supernatant was determined by spectrofluorimetry ($\lambda_{ex}/\lambda_{em}$ = 550/565 nm).

12. *In vivo* gene-silencing and anti-inflammatory efficiencies

ALI mice were sacrificed at 22 h post administration of NCs, and lung tissues were harvested and washed with PBS. The obtained lung tissues were homogenized using the Trizol reagent to collect total RNA, and the TNF-α mRNA level was determined by real-time PCR. In a parallel study, the lung tissues were homogenized using the passive lysis buffer containing protease inhibitor. The homogenate was centrifuged at 3,000 g and 4 °C for 10 min, and the supernatant was subjected to the determination of TNF-α and IL-6 levels using ELISA kits.

13. BALF collection and analysis

The BALF was collected at 22 h post NCs administration and was centrifuged at 3,000 g and 4 °C for 10 min. The supernatant was collected and subjected to the measurement of TNF-α and IL-6 levels using ELISA kits and total protein level using the BCA protein assay kit. The cell pellets were re-suspended in PBS to allow for total cell counting.

14. Measurement of myeloperoxidase level in lung tissues

The lung tissues were collected at 22 h post NCs administration and homogenized with the passive lysis buffer. The homogenate was centrifuged at 3,000 g and 4 °C for 10 min before determination of the myeloperoxidase level in the supernatant using a commercial assay kit.

15. Measurement of wet/dry weight ratio of lung tissues

The lung tissues were collected at 22 h post NCs administration, washed with PBS, and weighed to obtain the “wet” weight. The lung tissues were then dried at 80 °C for 72 h to obtain the “dry” weight, and the wet/dry weight ratio was calculated.

16. Blood gas analysis

Blood samples were obtained from the arterial carotids at 22 h post NCs administration, and were directly subjected to the measurement of PaO_2, $PaCO_2$, and pH by using the blood-gas analyzer (Radiometer, Shanghai, China).

17. Histological assessment

The lung tissues were harvested at 22 h post NCs administration, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 8-μm thickness, and stained with H&E before histological observation using an optical microscopy.

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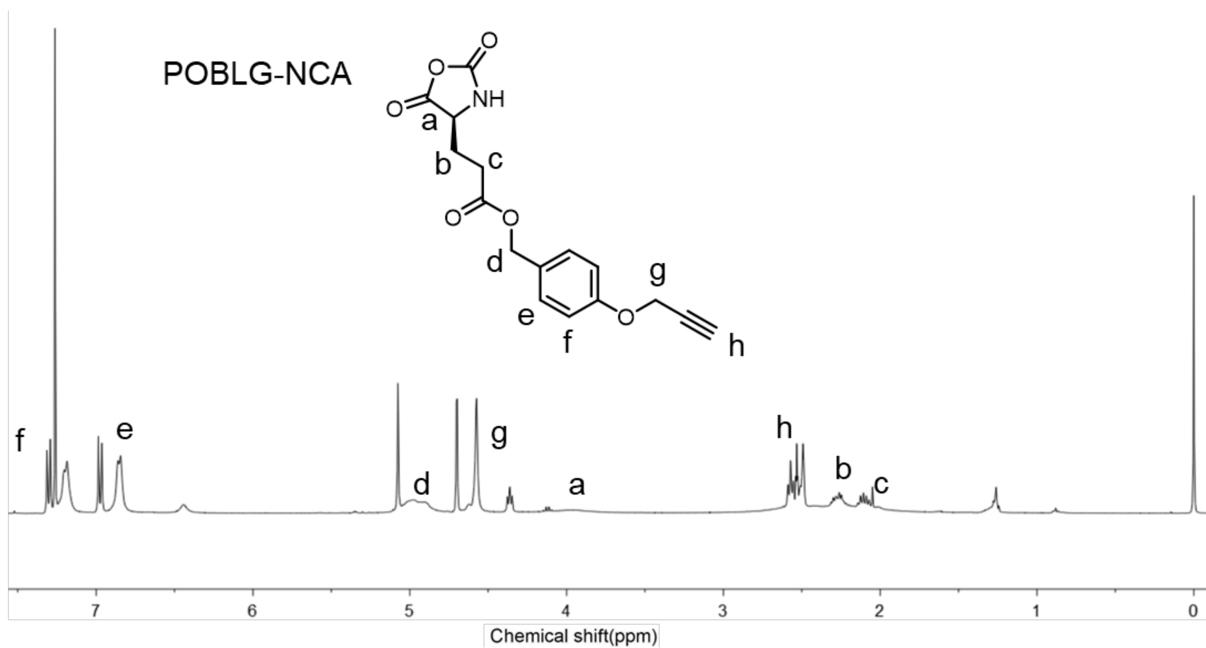

Fig. S1. ^{1}H NMR spectrum of POBLG-NCA ($CDCl_3$, 400 MHz).

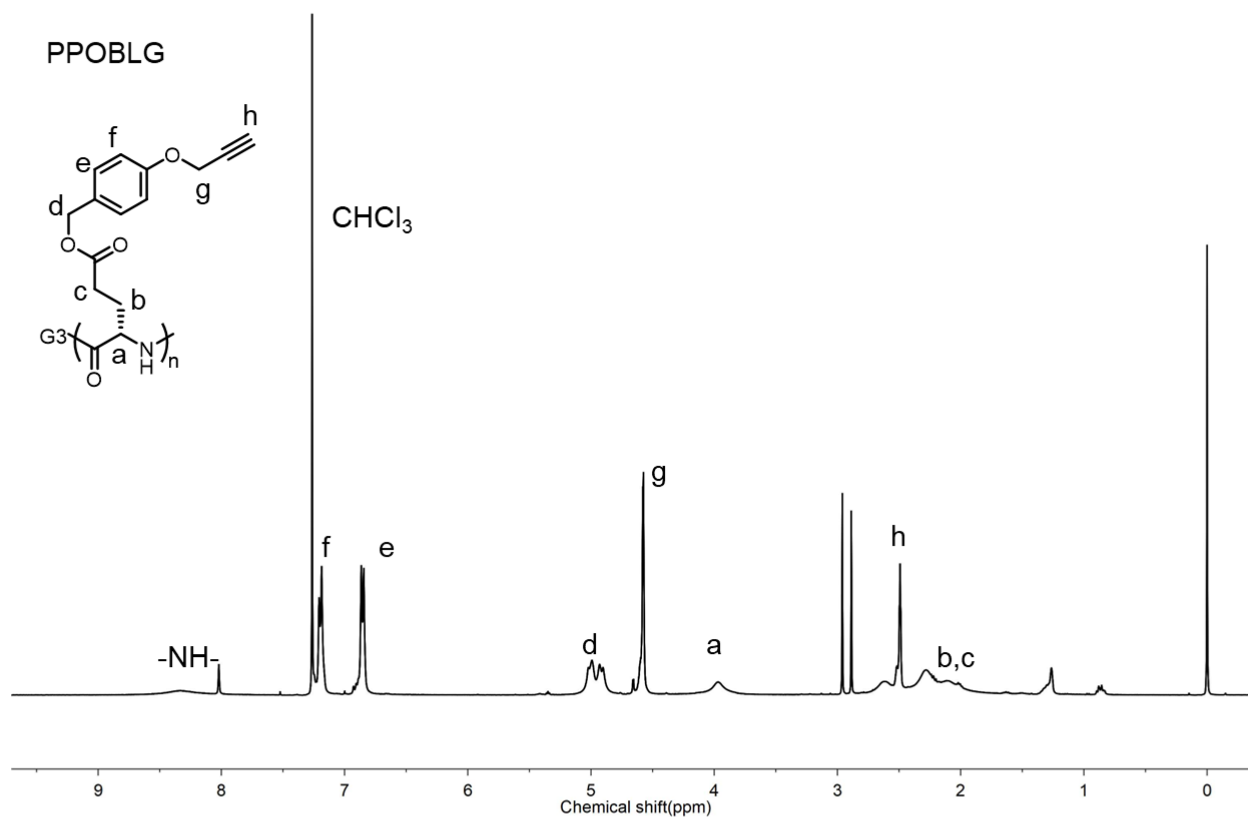

Fig. S2. ^{1}H NMR spectrum of PPOBLG ($CDCl_3$, 400 MHz).

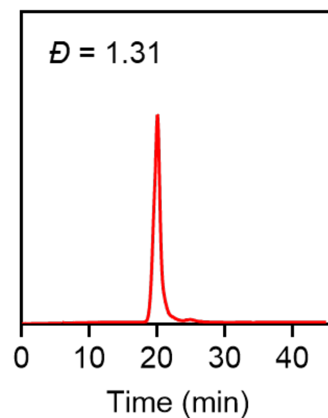

Fig. S3. GPC curve of PPOBLG using DMF containing 0.05 mol/L LiBr as the eluent phase at a flow rate of 1.0 mL/min.

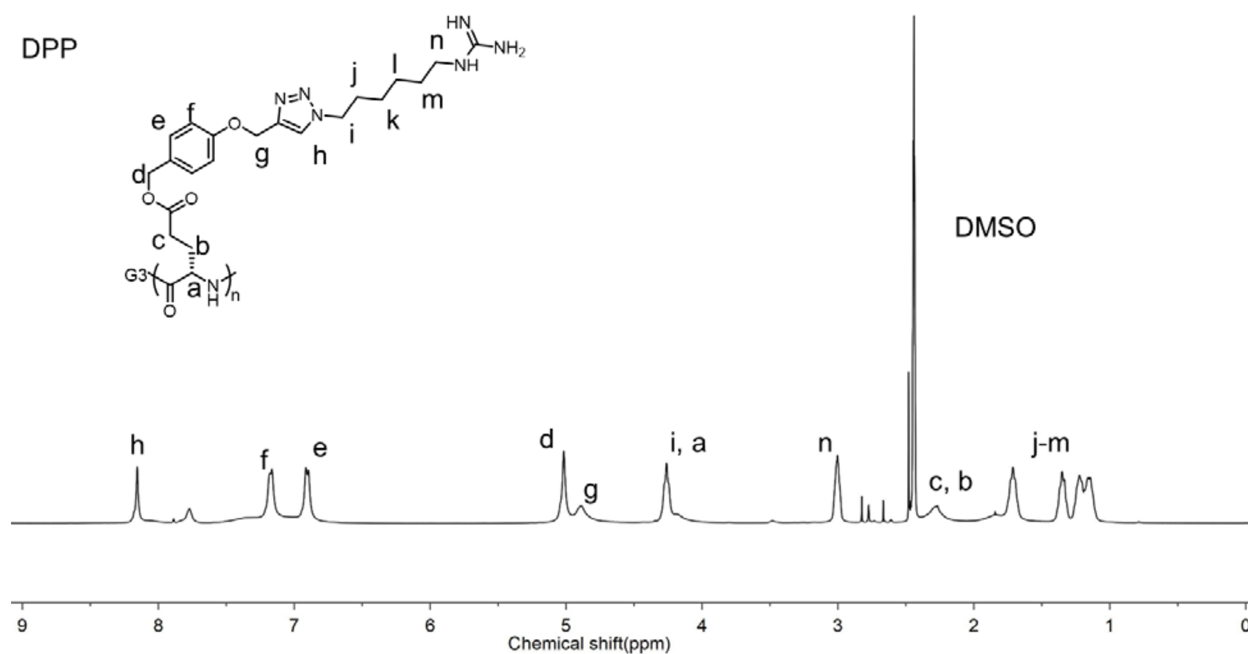

Fig. S4. ^{1}H NMR spectrum of DPP (DMSO-d_6, 400 MHz).

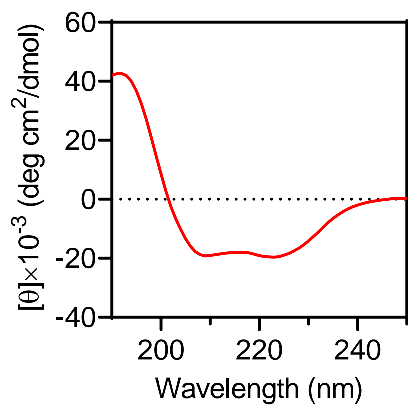

Fig. S5. CD spectrum of DPP (1 mg/mL) in deionized water.

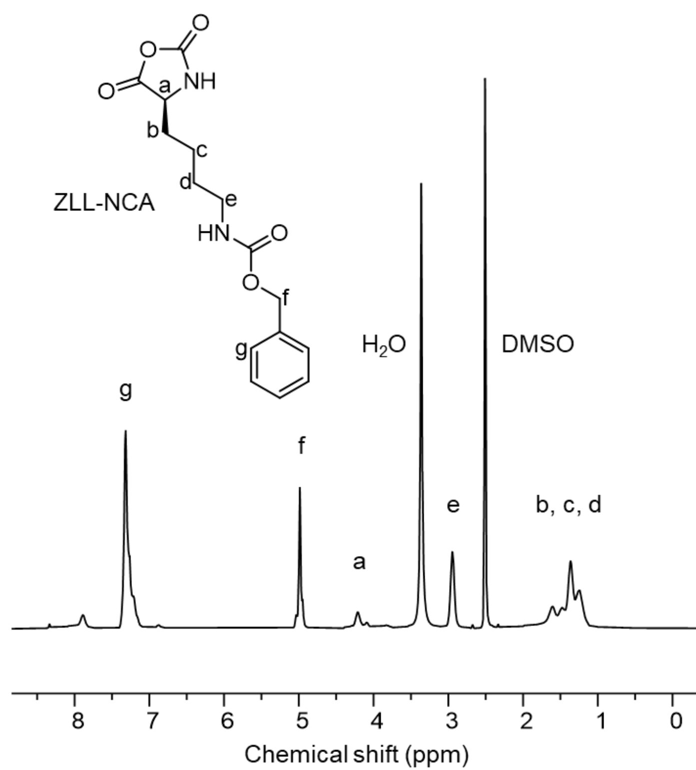

Fig. S6. ^{1}H NMR spectrum of ZLL-NCA ($CDCl_3$, 400 MHz).

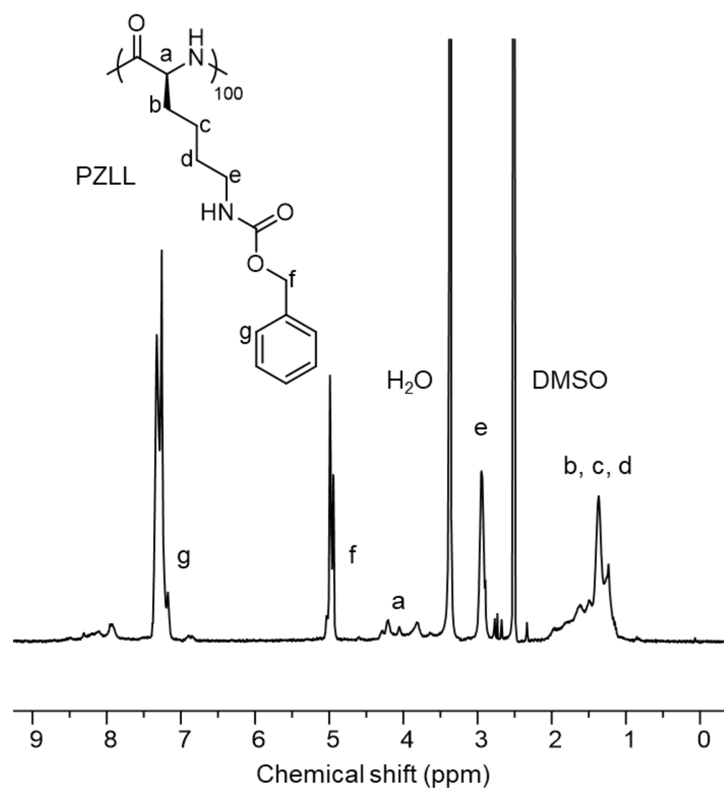

Fig. S7. ^{1}H NMR spectrum of PZLL ($CDCl_3$, 400 MHz).

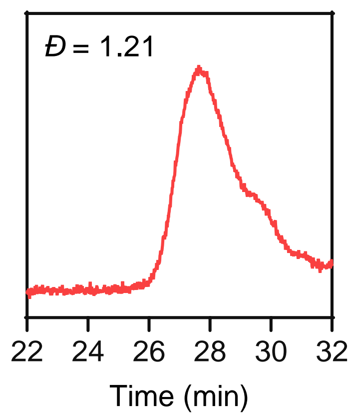

Fig. S8. GPC curve of PZLL using DMF containing 0.05 mol/L LiBr as the eluent phase at a flow rate of 1.0 mL/min.

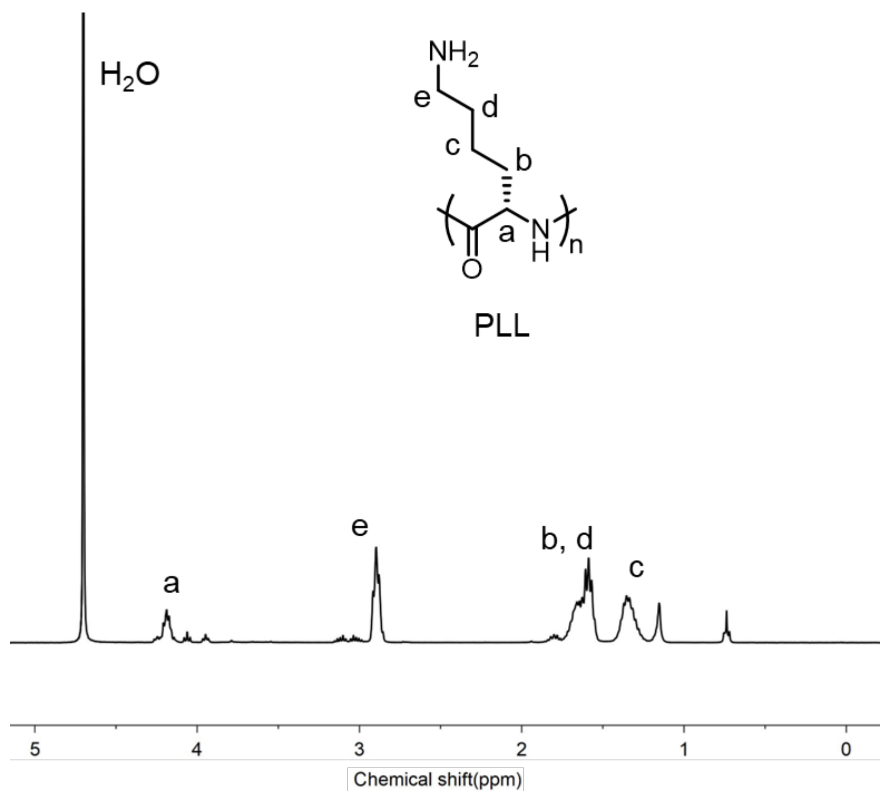

Fig. S9. ^{1}H NMR spectrum of PLL (D_2O, 400 MHz).

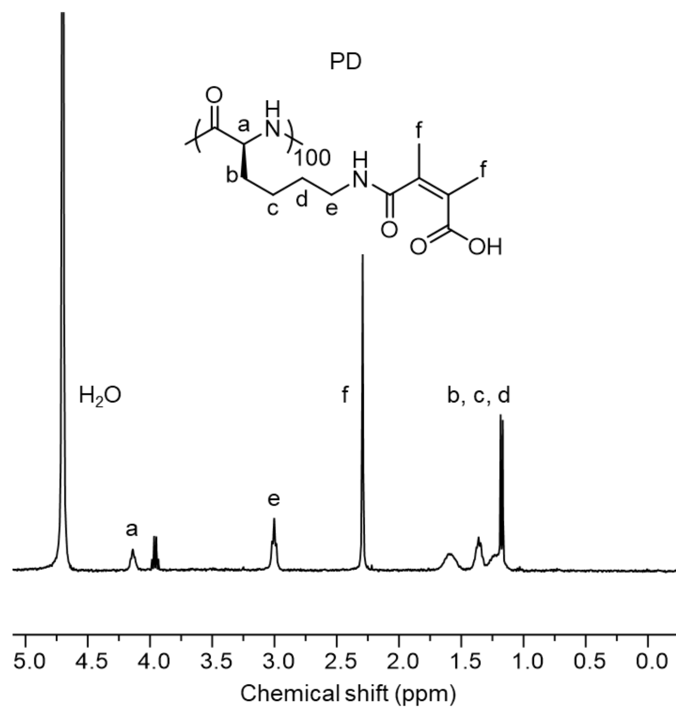

Fig. S10. ^{1}H NMR spectrum of PD (D_2O, 400 MHz).

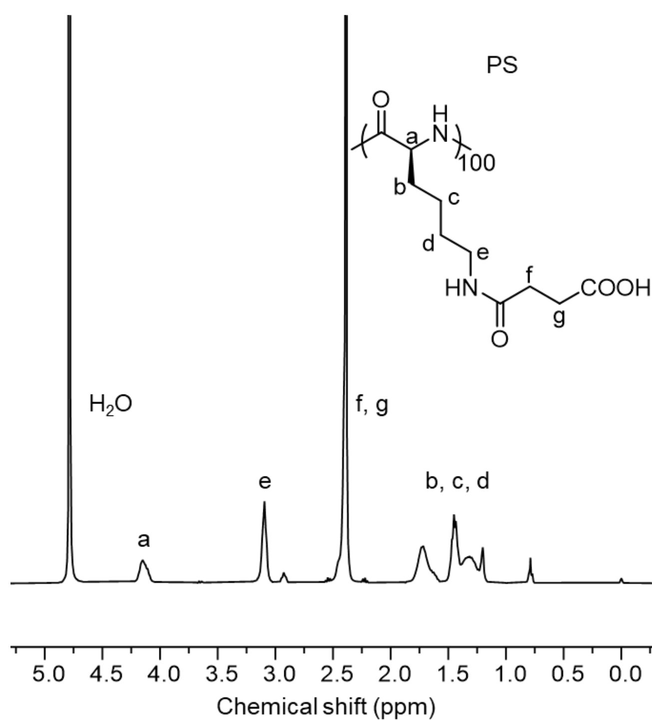

Fig. S11. ^{1}H NMR spectrum of PS (D_2O, 400 MHz).

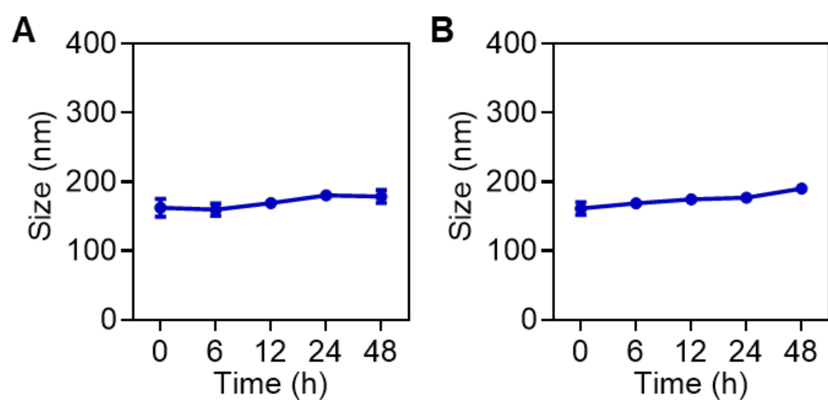

Fig. S12. Particle size of RDDsT NCs after incubation in PBS (A) or mucin solution (0.3% in saline, w/v, B) for different time (n = 3).

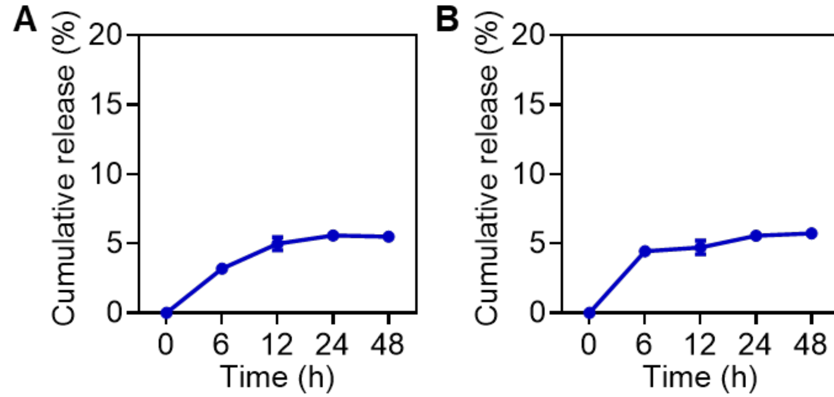

Fig. S13. Cumulative release of ^{Cy3}siTNF-α from RDDsT NCs after incubation in PBS (A) or mucin solution (0.3% in saline, w/v, B) for different time (n = 3).

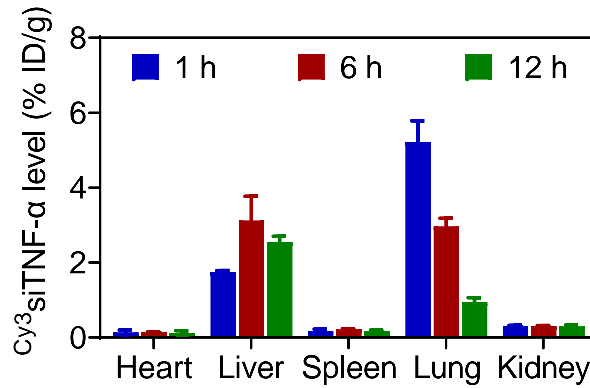

Fig. S14. ^{Cy3}siTNF-α level in the excised major organs (heart, liver, spleen, lung, and kidney) at 1, 6, and 12 h post intratracheal injection of RDDsT NCs containing ^{Cy3}siTNF-α (n = 3).

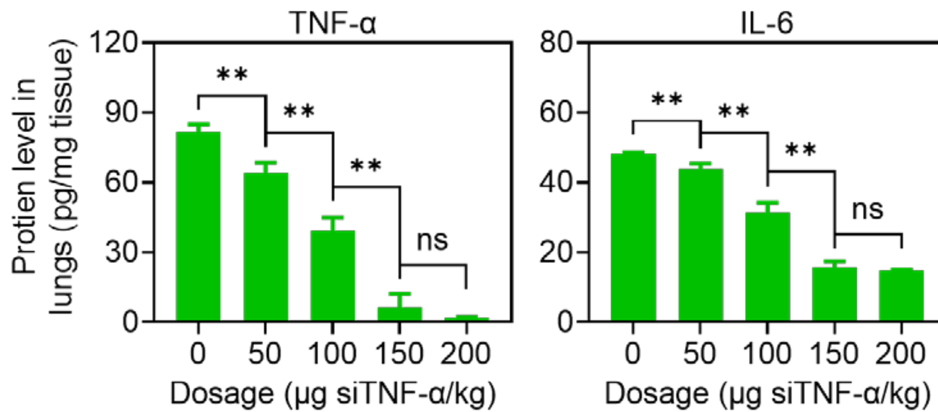

Fig. S15. Contents of TNF-α and IL-6 in lung tissues as determined by ELISA (n = 3). ALI mice were intratracheally injected with RDDsT NCs at 50, 100, 150, or 200 μg siTNF-α/kg.

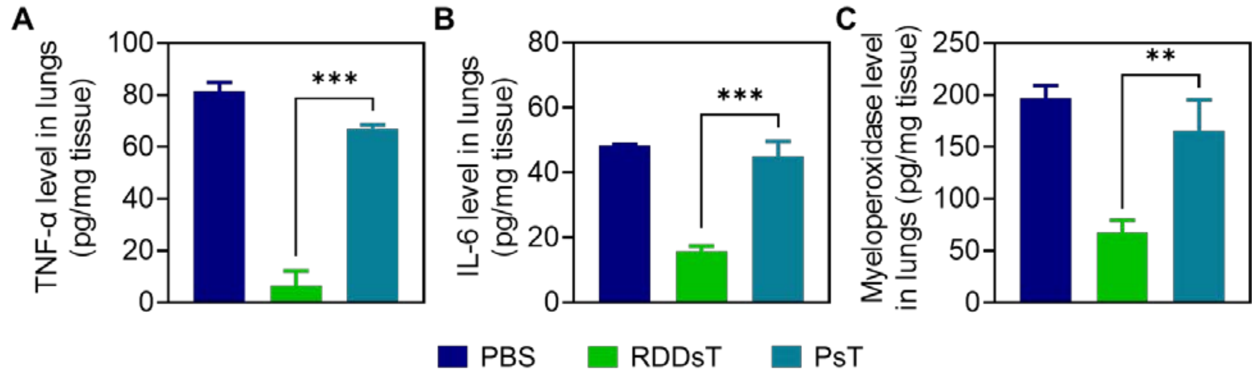

Fig. S16. Contents of TNF-α, IL-6, and myeloperoxidase in lung tissues (n = 3). ALI mice were intratracheally injected with RDDsT NCs or PsT NCs at 150 μg siTNF-α/kg.

Table S1. Full names and abbreviations of various NCs.

Full name	Abbreviation
DPP/siTNF-α	DsT
PD/siTNF-α	DDsT
PS/siTNF-α	SDsT
RBP/PD/DPP/siNC	RDDsC
RBP/PD/DPP/siTNF-α	RDDsT
RBP/PS/DPP/siTNF-α	RSDsT
PEI 25k/siTNF-α	PsT

Table S2. Sequences of siTNF-α and siNC.

	Sequence
siTNF-α sense	5'-GUCUCAGCCUCUUCUCAUUCCUGCT-3'
siTNF-α antisense	5'-AGCAGGAAmUGmAAmGAGGmCUGAmGACmAmU-3'
siNC sense	5'-UUCUCCGAACGUGUCACGUTT-3'
siNC antisense	5'-ACGUGACACGUUCGGAGAATT-3'

Table S3. Sequences of TNF-α and GAPDH primers.

	Sequence
TNF-α F	5'-CCCTCACACTCAGATCATCTTCT-3'
TNF-α R	5'-GCTACGACGTGGGCATCAG-3'
GAPDH F	5'-TTCACCACCATGGAGAAGGC-3'
GAPDH R	5'-GGCATGGACTGTGGTCATGA-3'