Supporting Information

Aminoglycoside-mimicking carbonized polymer dots for bacteremia treatment

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Experimental

Chemicals

L-Lysine (Lys), L-arginine (Arg), L-histidine (His), L-asparagine (Asn), L-tryptophan (Trp), L-cysteine (Cys), L-glutamic acid (Glu), L-threonine (Thr), D-mannose, hydrochloric acid, nitric acid, sodium chloride, monopotassium phosphate, disodium hydrogen phosphate, potassium chloride, and Luria broth (LB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and all cell culture media were purchased from Gibco BRL (Grand Island, NY, USA). Antibiotic-antimycotic, L-glutamine and nonessential amino acids (NEAAs) were obtained from Biowest (Lewes, UK). Alamar Blue reagent was purchased from BioSource (Camarillo, CA, USA). The SYTO Green Fluorescent Nucleic Acid Stain Assay Kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The cytotoxicity was quantified using a live/dead bacterial viability kit (Invitrogen, CA, USA). Phosphate-buffered saline (PBS; containing 137 $\times 10^{-3}$ M NaCl, 5.0 $\times 10^{-3}$ M KCl, 0.5 $\times 10^{-3}$ M $CaCl_2$, 1.0 $\times 10^{-3}$ M $MgCl_2$, 10 $\times 10^{-3}$ M Na_2HPO_4, and 2.0 $\times 10^{-3}$ M KH_2PO_4; pH 7.4) served to mimic physiological conditions. Five bacterial strains, *S. aureus* (ATCC 25923), *E. coli* (BCRC 12438), MRSA (ATCC 43300), *P. aeruginosa* (ATCC 10145), and *S. enterica* (ATCC 13076), were obtained from the Institute of Food Science (Hsinchu, Taiwan). Milli-Q ultrapure water (18.2 MΩ cm) from Millipore (Billerica, MA, USA) was used in all experiments.

Characterization of $CPDs_{Man/AA}$

TEM images of $CPDs_{Man/AA}$ were obtained using a Tecnai 20 G2 S-Twin transmission electron microscope (Philips/FEI, Hillsboro, OR, USA). Fluorescence (at excitation wavelengths in the range of 325–445 nm) and UV–Vis absorption spectra of $CPDs_{Man/AA}$ were recorded using a multi-mode microplate spectrophotometer (Synergy 4 Multi-Mode; Biotek Instruments, Winooski, VT, USA). The zeta potentials (ζ) of $CPDs_{Man/AA}$ were measured using a Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, UK). XPS was performed by an ES-CALAB 250 spectrometer (VG Scientific, East Grinstead, UK) with Al Kα X-ray radiation as the X-ray source for excitation. Binding energies were corrected using the C 1s peak at 284.6 eV as the standard. An Agilent Cary 640 FT-IR spectrometer (Santa Clara, CA, USA) was used to confirm the functional groups of the

$CPDs_{Man/AA}$. Elemental analysis of the $CPDs_{Man/AA}$ was performed by using a Vario EL cube analyzer (Elementar, Hanau, Germany) for N, C, H, and O.

Bacterial viability assay

The bacterial suspensions (1.0×10^7 CFU mL^{-1}) were treated with $CPDs_{Man/AA}$ (50 μg mL^{-1}) in 5.0 mM sodium phosphate solution (pH 7.4) at 37 °C under shaking for 60 min. The mixtures (1.0 mL) were then centrifuged (RCF 3,000 × *g*, 5 min, 37 °C) and washed thrice with sodium phosphate buffer (5 mM, pH 7.4) for the fluorescence observation of live and dead bacteria. Bacterial viability was determined by a LIVE/DEAD®Bac Light™ Bacterial Viability Kit (Molecular Probes). For each 1 mL of bacterial suspension, 3 μL of the dye mixture (SYTO 9: PI = 1:1) was added, and the dye-suspension mixtures were incubated at room temperature (25 °C) for 15 min in the dark. The mixtures were then centrifuged (RCF 3,000 × *g*, 5 min, 37 °C), washed twice with sodium phosphate buffer (pH 7.4; 1.0 mL), and dropped onto glass slides for fluorescence observation. The fluorescence images of the untreated bacteria were captured with the same procedure for bacterial labeling as with $CPDs_{Man/AA}$. The live and dead ratios of bacteria treated with $CPDs_{Man/AA}$ for different time durations were observed from the intensities of blue, green, and red fluorescence.

Agarose gel electrophoresis

E. coli suspension (10^9 CFU mL^{-1}) was centrifuged (RCF 3000 *g*, 5 min, 37 °C), washed thrice with 5.0 mM sodium phosphate buffer (pH 7.4), and resuspended in 1 mL Trizol reagent. After allowing to react for 3 min, 200 μL chloroform was added, mixed well and allowed to react for 20 min at –20 °C. Then, the solution was centrifuged at 12000 *g* for 10 min and washed twice with 75% ethanol. The RNA thus extracted was incubated with different concentrations (1–200 μg mL^{-1}) of $CPDs_{Man/Arg\text{-}190}$ for 1 h on ice. The samples were loaded in 2% agarose gel in 1X TAE buffer, and gel electrophoresis was carried out at 100 V for 25 min along with 1 KB DNA ladder (SMOBIO Technology Inc., Taiwan).

Cytotoxicity assays

Human embryonic kidney 293T cells (HEK-293T), human hepatocyte carcinoma (HepG2) and human umbilical vein endothelial cell (HUVEC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotic antimycotic (1%), L-glutamine (2 mM), and nonessential amino acids (1%) in 5% CO_2 at 37 °C. The cytotoxicity of $CPDs_{Man/Arg}$ was evaluated using PrestoBlue. Briefly, HaCaT cells were seeded in 96-well plates with 7.5×10^3 cells $well^{-1}$ and fed with DMEM containing 10% FBS. After 24 h of incubation in a humidified incubator containing 5.0% CO_2 at 37 °C, the medium was removed from each well, and the cells were further incubated with DMEM containing 10% FBS and $CPDs_{Man/Arg}$ at different concentrations and cultured for 24 h. After that, the culture medium was replaced with PrestoBlue and incubated for another 1 h at 37 °C. Finally, fluorescence was measured at an excitation/emission wavelength of 540/590 nm with a Synergy™ 4 multi-mode microplate spectrophotometer. The viability of the cells was expressed as a percentage of the control (untreated) cells (100%).

Hemolysis assays

Assays of the hemolysis induced by $CPDs_{Man/Arg}$ were performed according to a previously reported procedure.[1] Fresh blood samples from a healthy volunteer (female, 23 years old) were drawn from the blood vessels into tubes containing ethylenediaminetetraacetic acid and immediately (within 30 min of collection) centrifuged (RCF 3000 × *g*, 10 min, 4 °C) to remove the serum. The blood collection procedure was performed in compliance with relevant laws and institutional guidelines. Fresh RBCs were then washed three times with sterile isotonic PBS until the supernatant became transparent. RBCs were diluted with PBS to obtain an RBC stock suspension (4 vol% blood cells). The RBC stock suspension (500 µL) was added to each $CPDs_{Man/Arg}$ solution (1–100 µg mL^{-1}) in vials. After 1 h of incubation at 37 °C, each of the mixtures was centrifuged at an RCF of 1000 *g* for 10 min. Hemolysis activity was evaluated by absorption at 576 nm (OD_{576}) in the supernatant. Sterile isotonic PBS was used as a reference for 0% hemolysis. One hundred percent hemolysis was measured by adding ultrapure water to the RBC stock suspension. Deionized (DI) water was used as a control for 100% hemolysis.

$$\text{Hemolysis (\%)} = [(OD_{576,\ CPDsMan/Arg} - OD_{576,\ PBS})/(OD_{576,\ DI\ Water} - OD_{576,\ PBS})] \times 100$$

Measurement of intracellular ROS

RAW 264.7 cells were seeded at a density of 2.0×10^4 cells $well^{-1}$ in 96-well plates with DMEM containing 10% FBS. After 24 h, the RAW 264.7 cells in the culture wells were incubated with $CPDs_{Man/Arg\text{-}190}$ for 2 h. Then, the cell cultures were incubated in a medium containing 1.0 μg mL^{-1} LPS for 24 h. The intracellular accumulation of ROS was measured by the oxidative conversion of cell-permeable DCFH-DA to fluorescent 2',7'-dichlorofluorescein (DCF). The RAW 264.7 cells in the culture wells were incubated with 25 μM DCFH-DA solutions at 37 °C for 0.5 h. DCF fluorescence images were acquired from a fluorescence microscope, and fluorescence intensity was detected by a multi-mode microplate spectrophotometer.

In vivo treatment of LPS-induced sepsis

Six-week-old male BALB/c mice were purchased from BioLASCO (Taipei, Taiwan) and raised in the animal center of National Taiwan Ocean University (Keelung, Taiwan). Then, LPS solution (200 μL; 5 mg kg^{-1}) in presterilized saline was intraperitonealy injected into the mice (6 mice per group). After 2 h of administration, the mice were treated (intraperitoneal injection) with PBS (as a negative control) and 5 mg kg^{-1} $CPDs_{Man/Arg\text{-}190}$ every 8 h for 3 days. The survival rate was recorded daily for 6 days or more.

Table S1. Elemental composition of $CPDs_{Man/AA}$.

CPDs	Elemental composition				
	O (%)[a]	N (%)[a]	C (%)[a]	H (%)[a]	Cl (%)[b]
$CPDs_{Man/Lys-130}$	26.9	12.9	40.1	8.1	15.2
$CPDs_{Man/Arg-130}$	24.6	23.3	36.7	7.4	15.3
$CPDs_{Man/His-130}$	27.5	17.8	37.5	5.6	11.1
$CPDs_{Man/Lys-160}$	25.7	12.9	41.3	8.3	13.5
$CPDs_{Man/Arg-160}$	28.9	21.3	35.7	7.6	14.8
$CPDs_{Man/Lys-190}$	25.3	12.8	42.3	8.6	14.1
$CPDs_{Man/Arg-190}$	25.1	21.6	35.2	7.9	15.4

[a] determined by elemental analysis (EA).

[b] determined by inductively coupled plasma mass spectrometry (ICP-MS).

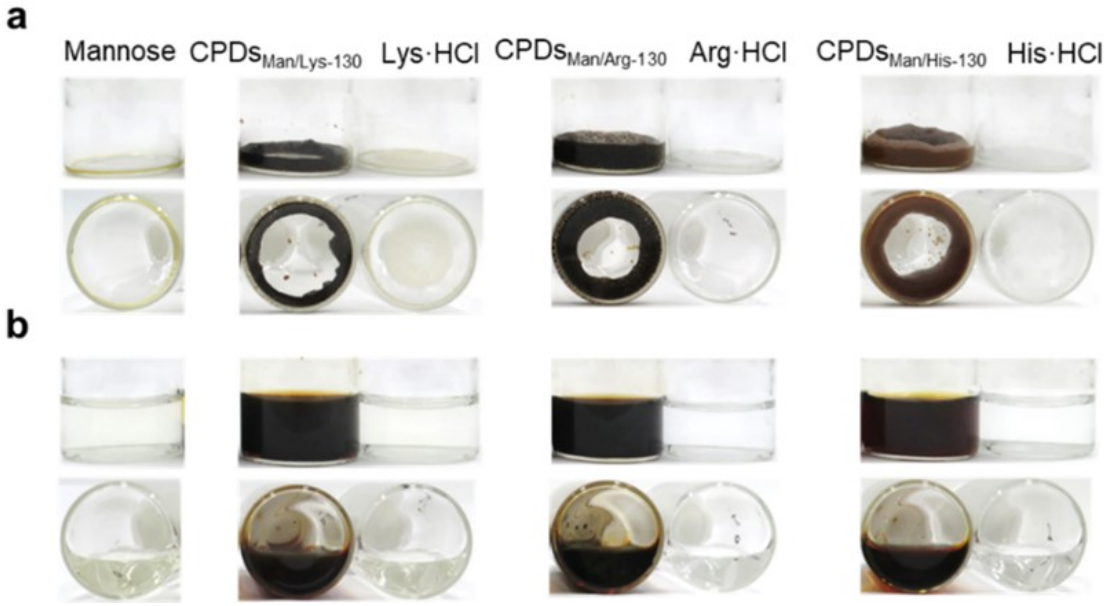

Fig. S1. Photographs of $CPDs_{Man/AA}$. (a) As-prepared products and (b) products dissolved in deionized water (5.0 mL) after the pyrolysis of mannose (Man), basic amino acids [lysine (Lys)·HCl, arginine (Arg) ·HCl or histidine (His)·HCl] or their mixtures at 130 °C for 3 h.

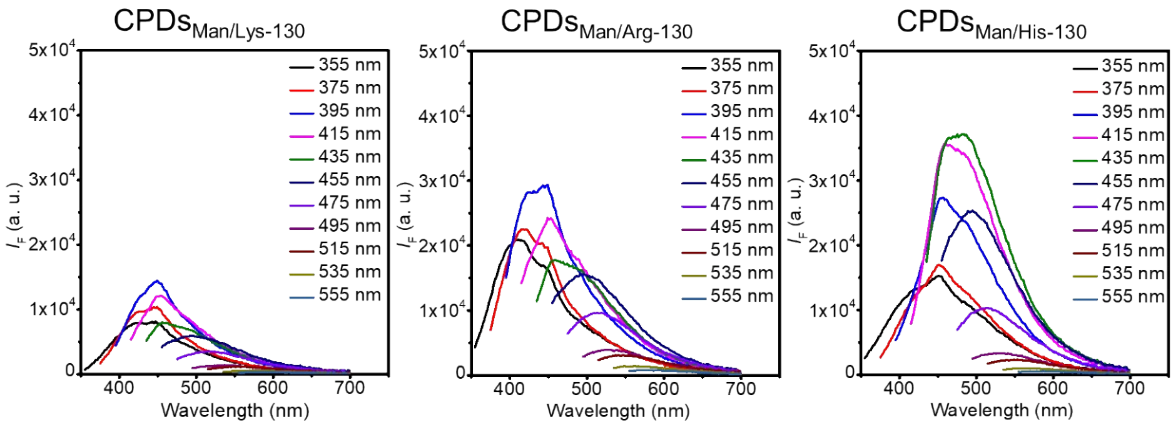

Fig. S2. Excitation-dependent fluorescence emission spectra of $CPDs_{Man/AA}$.

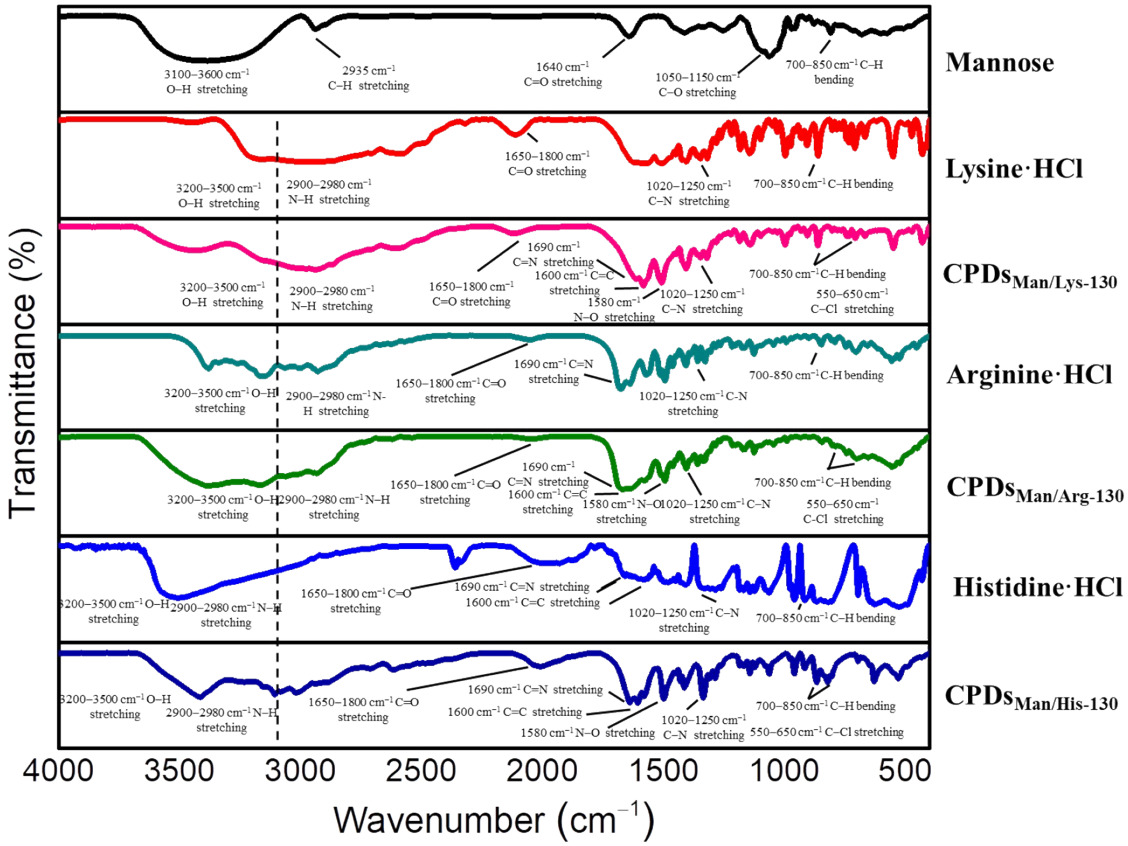

Fig. S3. FT-IR spectra of mannose, amino acids, and purified $CPDs_{Man/AA–130}$.

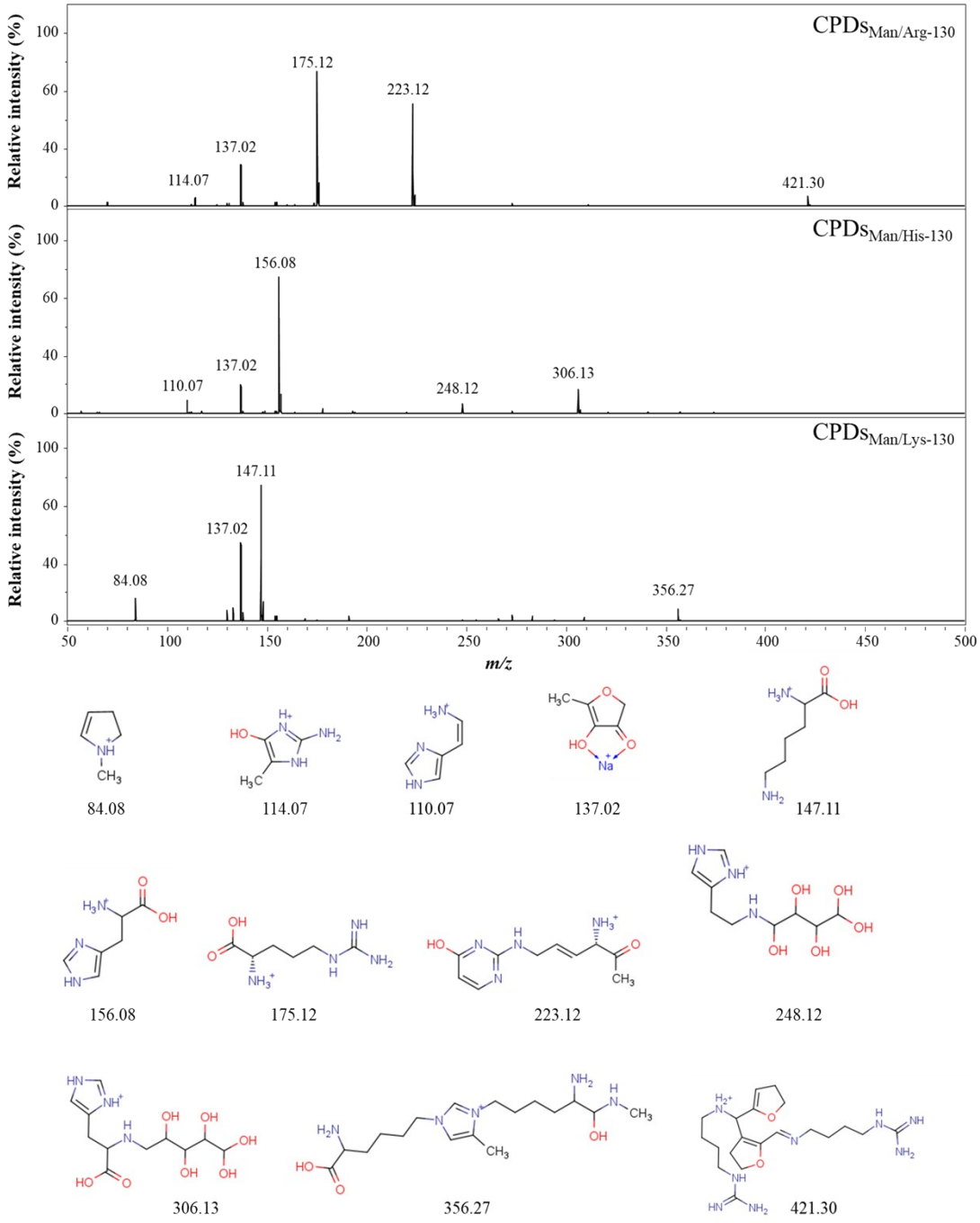

Fig. S4. LDI-MS spectra of purified $CPDs_{Man/AA-130}$.

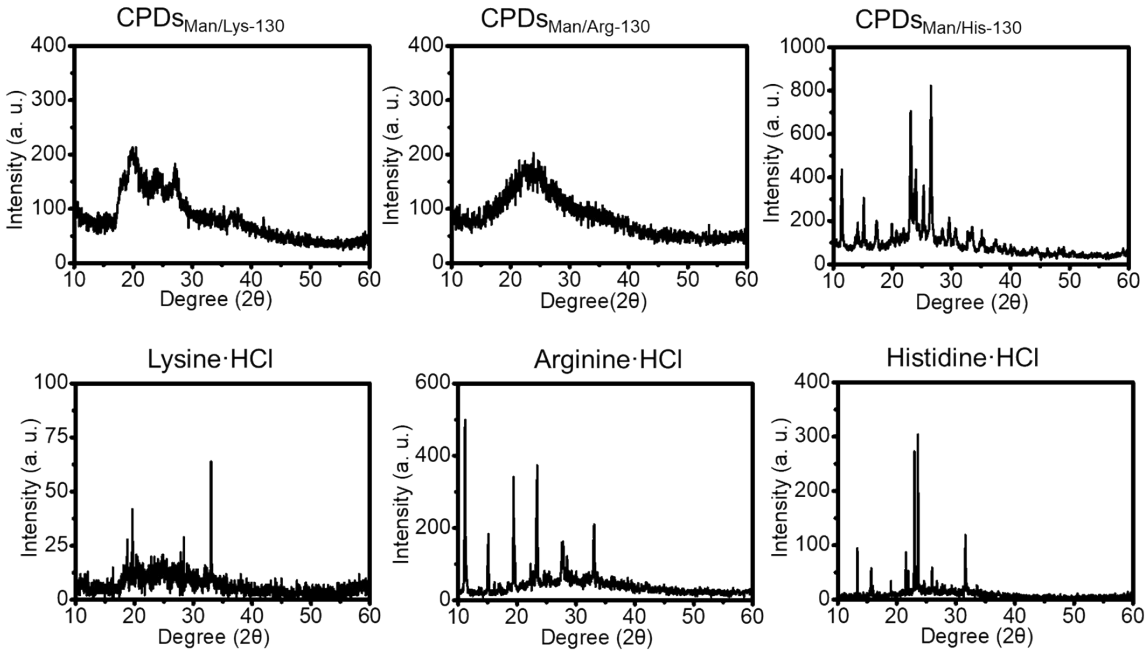

Fig. S5. XRD spectra of amino acids and purified $CPDs_{Man/AA-130}$.

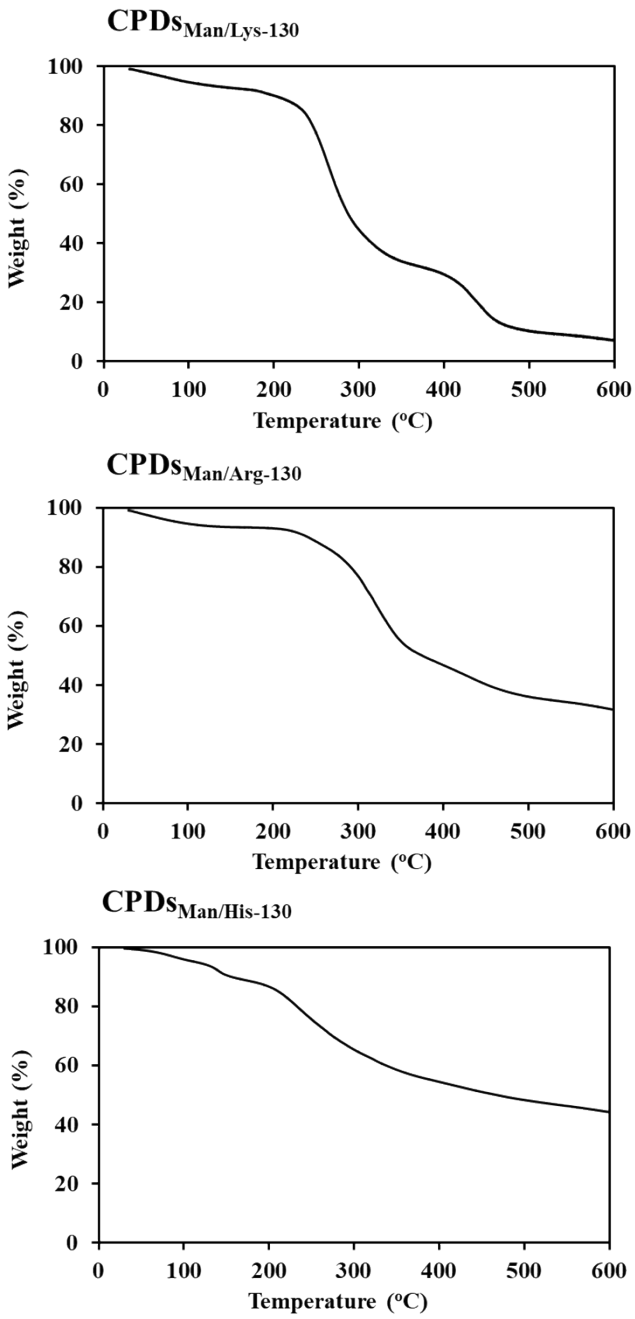

Fig. S6. TGA analysis of purified $CPDs_{Man/AA-130}$ with the heating rate of 5 °C min^{-1} under N_2 atmosphere.

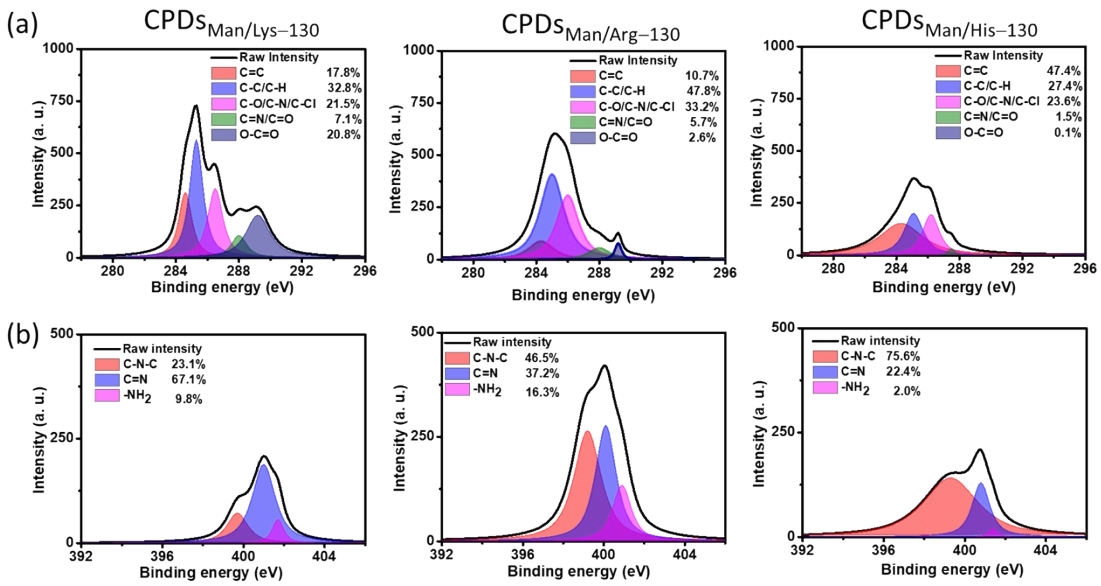

Fig. S7. XPS of purified CPDs$_{Man/AA-130}$ (a) C 1s and (b) N 1s spectra.

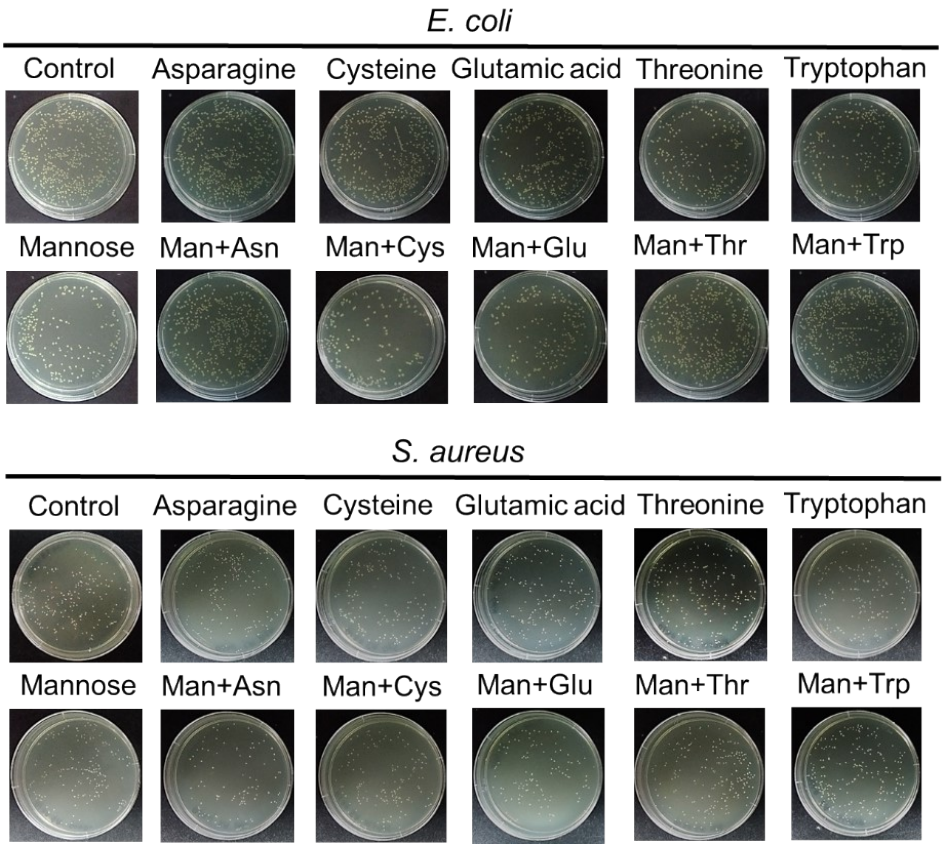

Fig. S8. Colony formation of *E. coli* and *S. aureus* on LB agar plates: untreated (control) and treated with 5.0 μg mL^{-1} of carbon materials prepared from amino acids or the mixture of mannose and amino acids (asparagine, cysteine, glutamic acid, threonine or tryptophan) pyrolyzed at 130 °C for 3 h.

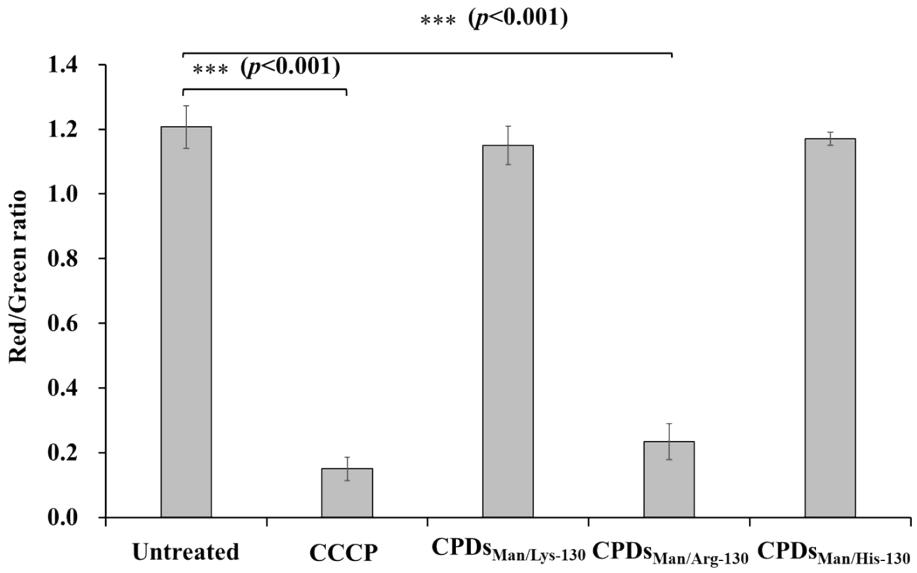

Fig. S9. Fluorescence intensity ratio of $DiOC_2(3)$ at 590 and 520 nm (Red/Green ratio) of the *S. aureus* (1.0×10^7 CFU mL^{-1}) solution incubated without (untreated), with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (as a control) or 50 μg mL^{-1} of $CPDs_{Man/Lys-130}$, $CPDs_{Man/Arg-130}$ or $CPDs_{Man/His-130}$. The reaction was carried out in 5.0 mM sodium phosphate solution (pH 7.4) containing 1.0 mM $MgCl_2$ and 10 mM KCl for 10 min. The error bars represent the standard deviation of three repeated measurements. Error bars represent the standard deviation of three repeated measurements.

The $DiOC_2(3)$ loaded cells fluoresce red (emission 590 nm), which is consistent with the presence of $DiOC_2(3)$ aggregates and intact membrane potential. Cells treated with the protonophore CCCP fluoresce green (emission 520 nm) are consistent with the presence of monomeric $DiOC_2(3)$ and the dissipation of membrane potential.

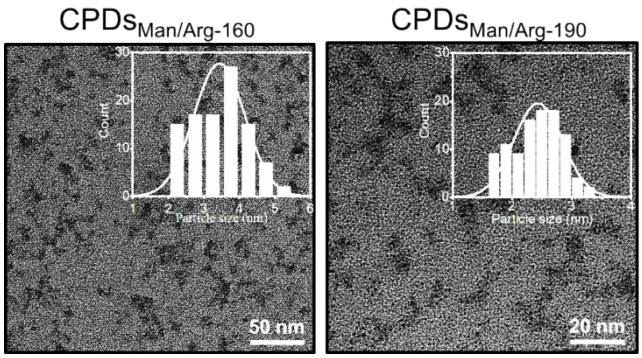

Fig. S10. TEM images of $CPDs_{Man/Arg-160}$ and $CPDs_{Man/Arg-190}$.

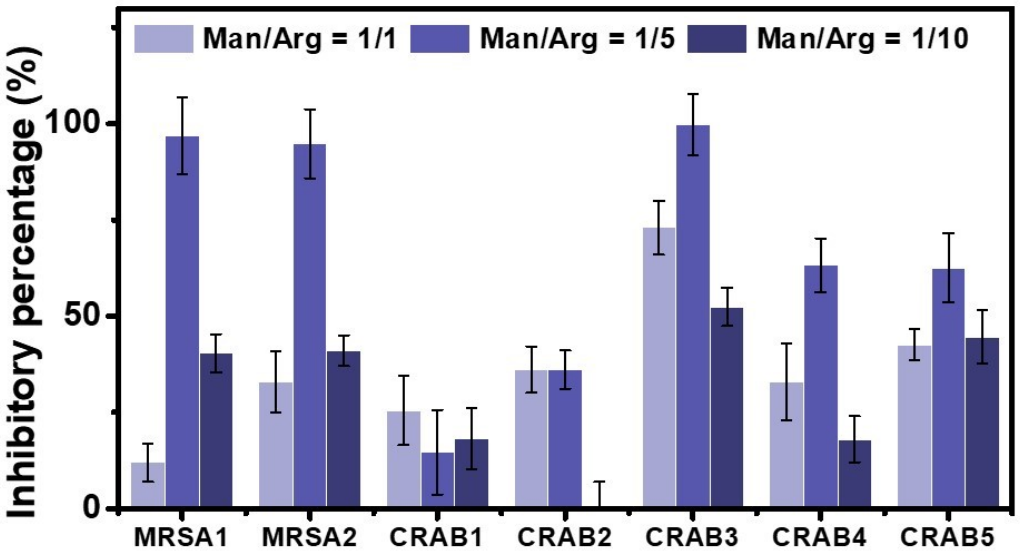

Fig. S11. Bacterial inhibition (%) of Gram-positive bacteria (MRSA1 and MRSA2) and Gram-negative bacteria (CRAB 1–5) after incubation with $CPDs_{Man/Arg\text{-}190}$ which were synthesized from different ratio of mannose and arginine (Man/Arg = 1/1, 1/5, and 1/10) at the concentration of 0.1 μg mL^{-1} in 5.0 mM sodium phosphate buffer (pH 7.4) for 3 h. Error bars represent the standard deviation of three repeated measurements.

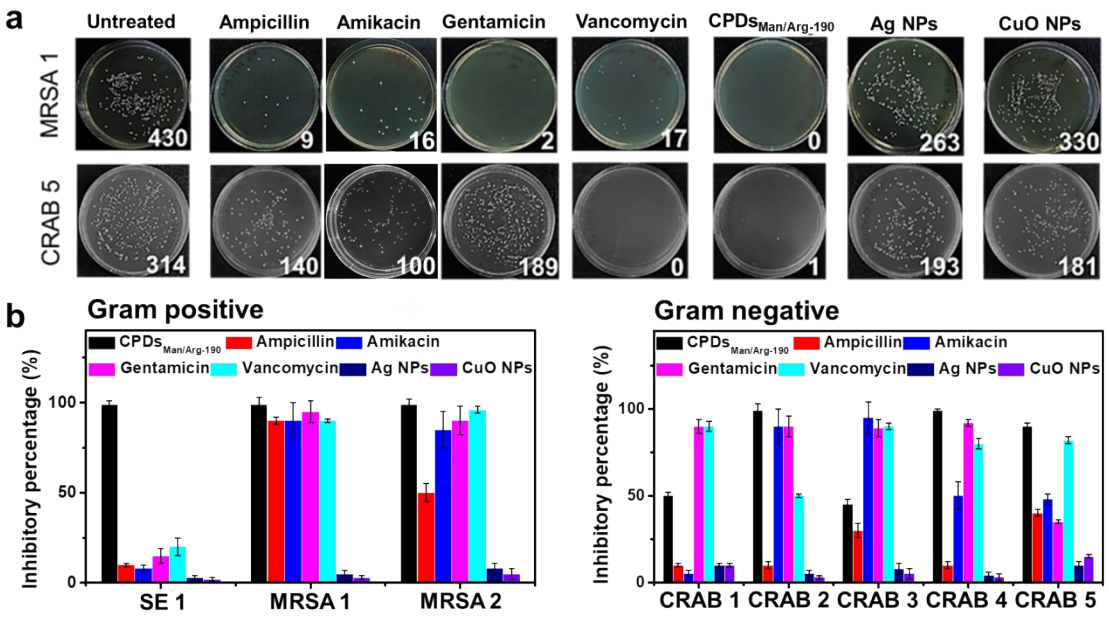

Fig. S12. Antibacterial assay of antibiotics and nanomaterials in human plasma. (a) Representative colony formation of MRSA 1 and CRAB 1 suspensions (10^4 CFU mL^{-1}) untreated or treated with 100 μg mL^{-1} antibiotics, $CPDs_{Man/Arg-190}$, Ag NPs, or CuO NPs dispersed in 50% v/v plasma solution for 12 h. The inset values show the CFUs of the corresponding assay. (b) Bacterial inhibition (%) of Gram-positive bacteria (SE1, MRSA 1, MSSA 1) and Gram-negative bacteria (CRAB 1–5) after incubation with antibiotics (100 μg mL^{-1}) or nanomaterials (100 μg mL^{-1}) in 50% v/v plasma solution for 12 h. Error bars in (b) represent the standard deviation of three repeated measurements.

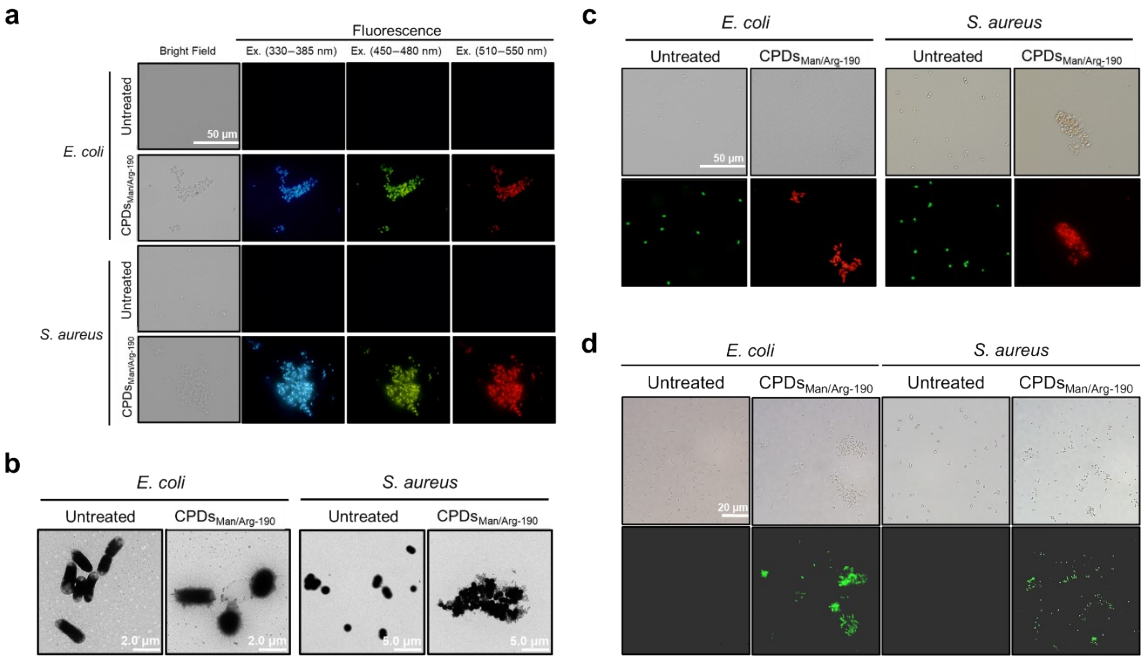

Fig. S13. Microscopic images of *E. coli* and *S. aureus* after treating with $CPDs_{Man/Arg\text{-}190}$. (a) Bright-field and excitation-dependent fluorescent images of *E. coli* and *S. aureus* (10^7 CFU mL^{-1}) after incubation with $CPDs_{Man/Arg\text{-}190}$ (50 μg mL^{-1}) for 1 min in 5.0 mM sodium phosphate buffer (pH 7.4). For fluorescence images, UV (330–385 nm), blue (450–480 nm), and green (510–550 nm) light were used to excite CPDs. (b) TEM images and (c) bright-field and fluorescence images from SYTO9/PI staining of *E. coli* and *S. aureus* before and after treatment with $CPDs_{Man/Arg–190}$ (50 μg mL^{-1}) for 1 h. SYTO9 and PI dyes exhibit green and red fluorescence, corresponding to live and dead cells, respectively. (d) Bright-field and fluorescent images of *E. coli* and *S. aureus* (10^7 CFU mL^{-1}) staining with DCFH-DA to assess the level of ROS after treatment with $CPDs_{Man/Arg\text{-}190}$ (50 μg mL^{-1}) for 1 h in 5.0 mM sodium phosphate buffer (pH 7.4). A negative control is conducted without $CPDs_{Man/Arg\text{-}190}$.

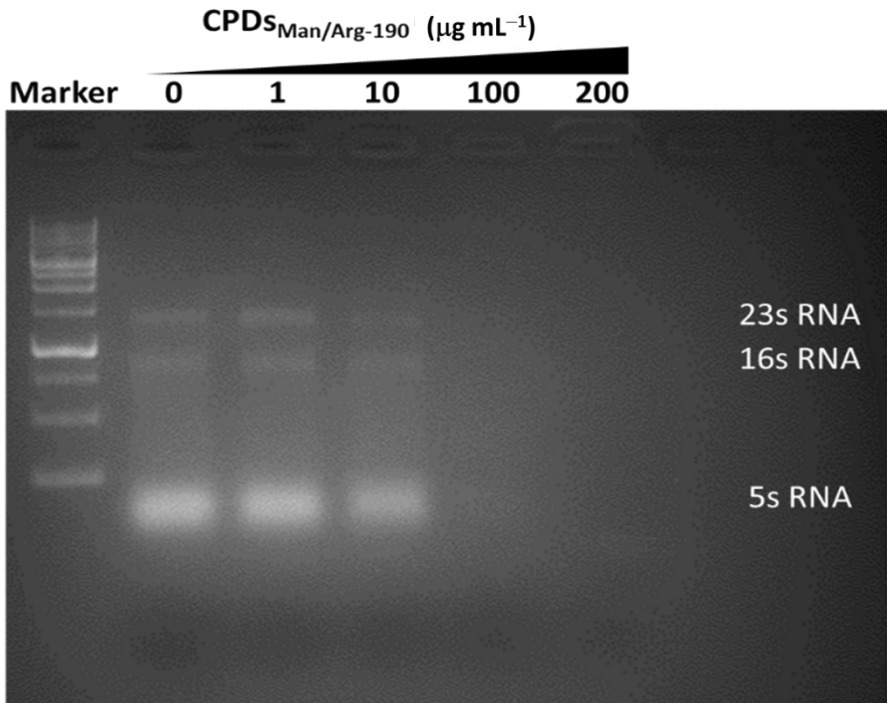

Fig. S14. Agarose gel electrophoresis to demonstrate the binding of $CPDs_{Man/Arg\text{-}190}$ to 5S, 23S and 16S rRNA.

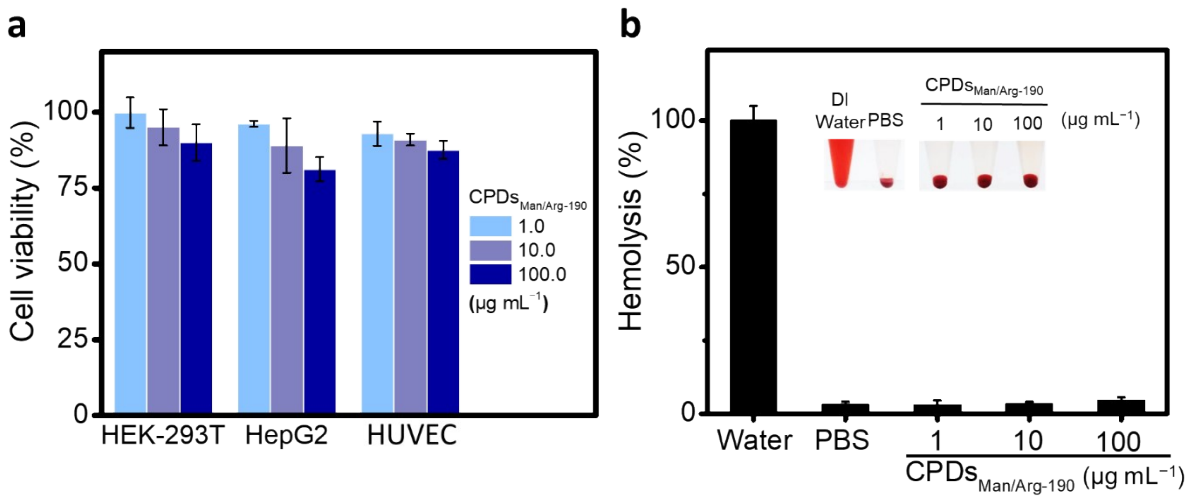

Fig. S15. Cell viability and hemolysis assays. (a) Cell viability of HEK-293T, HepG2 and HUVEC (10^4 cells $well^{-1}$) after treatment with $CPDs_{Man/Arg-190}$ (1–100 μg mL^{-1}) for 24 h. (b) Hemolytic activities of $CPDs_{Man/Arg-190}$ toward RBCs (4%) in PBS. PBS- and DI water-treated RBCs were used as negative and positive controls, respectively. Error bars show the standard deviation of three repeated experiments. Photographs in (b) represent the corresponding solutions.

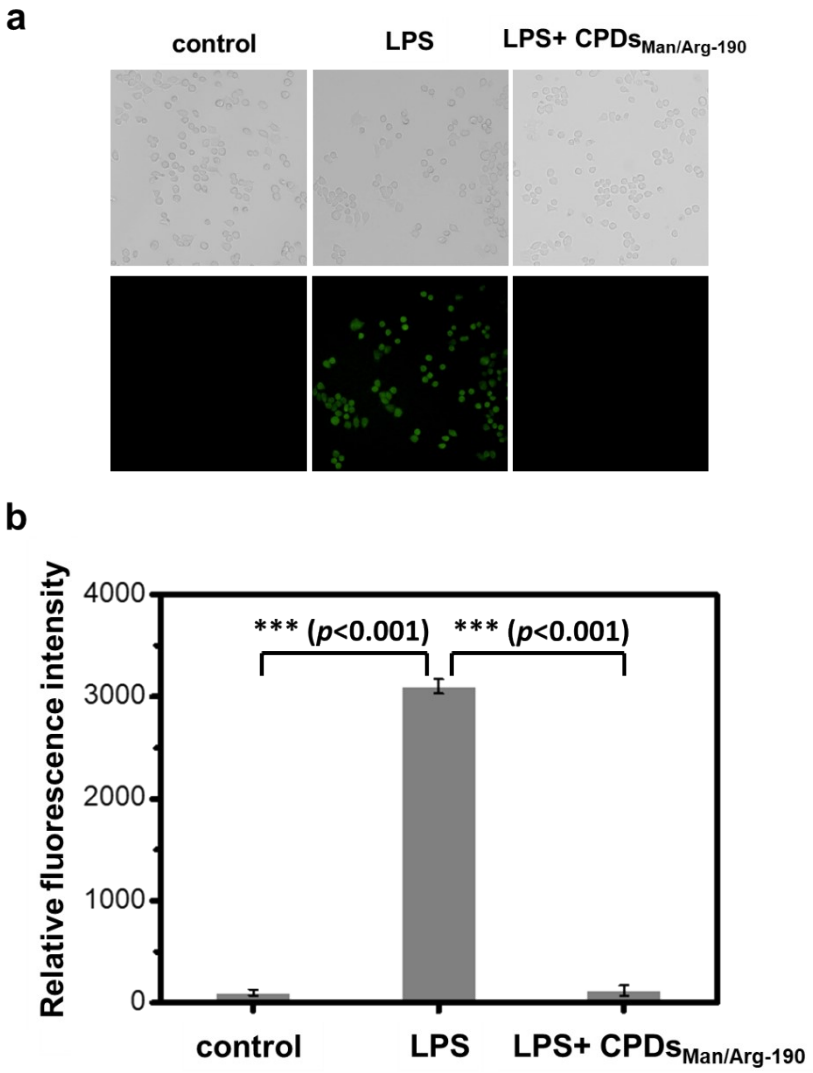

Fig. S16. Measurement of $CPDs_{Man/Arg-190}$ suppresses the LPS-induced intracellular ROS in macrophage cells. (a) Bright-field and fluorescence images and (b) green fluorescence intensity (510–550 nm) of RAW cells and LPS-stimulated RAW 264.7 cells followed by treatment with $CPDs_{Man/Arg-190}$ for 2 h and then staining with DCFH-DA. Values in (b) are the mean ± standard deviation (n = 4).

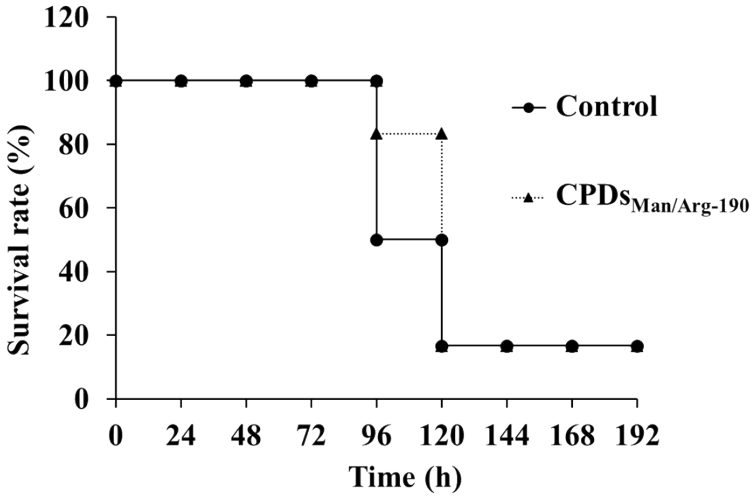

Fig. S17. Survival rates of mice intravenously injected with PBS (as a control) or $CPDs_{Man/Arg\text{-}190}$ (5 mg kg^{-1}) after LPS-induced systematic inflammation.

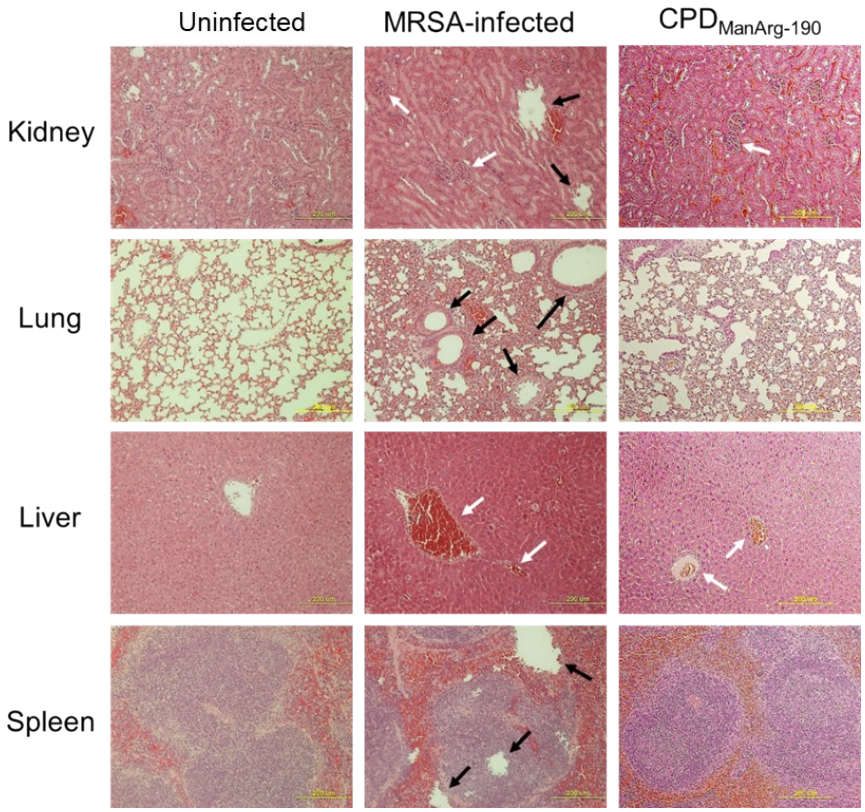

Fig. S18. Histological images of kidney, lung, liver, and spleen tissue stained by H&E from uninfected mice, mice infected by MRSA, and mice infected by MRSA and then treated with $CPDs_{Man/Arg-190}$. Tissue samples were collected on the first day postinfection. White arrows indicate severe inflammation and fibrosis, and the black arrows indicate cellular swelling and large lipid vacuoles. Scale bars are 200 μm.

Reference

1. C. T. Pham, D. G. Thomas, J. Beiser, L. M. Mitchell, J. L. Huang, A. Senpan, G. Hu, M. Gordon, N. A. Baker, D. Pan, G. M. Lanza and D. E. Hourcade, *Nanomedicine*, 2014, **10**, 651–660.