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Supplementary Information for

Selective treatment of intracellular bacterial infections using host cell-targeted biorthogonal nanozymes

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Table of contents

Experimental methods

Materials. All chemicals and materials for experiments were obtained from Sigma Aldrich (USA) or Fisher Scientific (USA). Further purification was not performed unless otherwise indicated. Chloroauric acid used for gold nanoparticle synthesis was purchased from Strem Chemicals Inc. (Newburyport, MA, USA).

RAW 264.7 cells (ATCC TIB-71) and HEPG2 cells (ATCC HB-8065) were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM; for RAW 264.7 cultures), Eagle's Minimum Essential Medium (EMEM; for HEPG2 cultures), fetal bovine serum (FBS; Fisher Scientific) were used in cell culture. *Salmonella enterica* subsp*. enterica* serovar Typhimurium (ATCC 29630) was purchased from ATCC and cultured using lysogeny broth (LB; Fisher Scientific). *Lactobacillus* species used were obtained from probiotic BioK^{+} and cultured using *Lactobacilli* De Man, Rogosa and Sharpe (MRS) broth (Fisher Scientific).

Synthesis of mannose-decorated gold nanoparticles (Man-AuNPs). **AuNPs** used in this study were synthesized as reported previously.[1] Briefly, the Brust-Schiffrin two-phase synthesis method was used to synthesize 1-pentanethiol protected 2 nm **AuNPs**. A carboxylate-terminated ligand (COOH ligand) was synthesized using previously reported procedures.[1,2] Murray place-exchange method was followed to attach the ligand to the **AuNP** core. 30 mg of pentanethiol-conjugated **AuNPs** were dissolved in 4 mL distilled dichloromethane (DCM) and mixed with 90 mg of COOH ligand dissolved in 4 mL of 1:1 DCM:methanol, and stirred for 3 days at room temperature under nitrogen environment, followed by removal of DCM and methanol under reduced pressure. Excess ligands, pentanethiol, acetic acid, and other salts present in the **AuNP** solution were removed by washing with hexane thrice and DCM twice, followed by re-dispersion in de-ionized water and dialysis (membrane molecular weight cut-off = 10,000) for 3 days. After dialysis, the particles were filtered through a syringe filter to remove impurities [Amicon (USA), Ultra 4, 10K]. The carboxylate **AuNPs** were then post-functionalized with mannose to give **Man-AuNPs** by adding 1 mM mannose amine, 3 mM N-Hydroxysuccinimide (NHS) and 3 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) on an ice bath. The reaction was allowed to proceed overnight and the mixture was then collected and washed with Milli-Q water multiple times using ultracentrifugal filters (Amicon Ultra 4, MWCO=10 KDa, pore size = 0.2 μm) at 4,000 rpm to remove the excess reactants. The final AuNPs solution was filtered through a 0.2 μm polyethersulfone (PES) membrane filter and then characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

Zeta potential characterization of Man-AuNP. Zeta potential was measured in deionized (DI) water using a Malvern Zetasizer Nano ZS instrument.

DLS and TEM characterization of Man-AuNP. Hydrodynamic diameters of **Man-AuNPs** and mannose nanozymes (**Man-NZs**) were measured by DLS in DI water using a Malvern Zetasizer Nano ZS instrument by using a measurement angle of 173° (backscatter). Data were analyzed

by the "multiple narrow modes" (high resolution) method based on non-negative-least-squares (NNLS).

TEM imaging samples were prepared separately by placing a droplet of 1 μM Man-AuNP or Man-NZ solution onto a 300-mesh Cu-grid coated with carbon film. The samples were analyzed using a JEOL JEM-2000FX (located in the Institute of Applied Life Sciences) with an accelerating voltage of 200 keV electron microscope. These characterization data indicated that there was no aggregation before or after catalyst encapsulation.

Carbohydrate density determination. A freshly prepared anthrone solution in concentrated H_2SO_4 (0.5 wt %, 1 mL) was added to various concentrations of D-mannose in water (0.5 mL) in an ice bath with stirring. The solution was then heated to 100 °C and stirred for 10 min. After cooling to room temperature, the absorbances of the resulting solutions were measured at 620 nm on a Spectramax M2 Microplate Reader (Molecular Devices, San Jose, CA, USA). The data were plotted against the concentrations of D-mannose and used as the calibration curve for the calculation of ligand density on **Man-AuNPs**. Ligand density experiments for **Man-AuNPs** were carried out by dissolving freshly-prepared D-mannose-conjugated **AuNPs** (5 μM) in 0.5 mL Milli-Q water, and treating solutions with anthrone/H_2SO_4 following the same protocol described above. The final data shown are mean values obtained from three measurements. The non-functionalized **COOH-AuNPs** were treated with anthrone/H_2SO_4 in the same manner, and the absorbance at 620 nm was used as the background deducted from the total signal measured from D-mannose-conjugated **AuNPs**. The density of immobilized D-mannose was then determined using the calibration curve.

Catalyst encapsulation in AuNP monolayer to fabricate mannose nanozyme (Man-NZ). 2 mL of iron (III) tetraphenyl porphyrin (**FeTPP**) solution (2 mg/mL) in tetrahydrofuran (THF) was mixed with an equal volume of aqueous **Man-AuNP** solution (10 μM) and stirred for 10 min. The organic layer was slowly evaporated to induce the encapsulation of **FeTPP** into the NP monolayer, with excess catalyst precipitating from solution. Excess **FeTPP** was removed by 0.22 μm PES membrane filter. Then the dispersion was washed with ultracentrifugal filters (Amicon Ultra 4, MWCO=10 KDa, pore size = 0.2 μm), washing with Milli-Q water multiple times at 4,000 rpm until no color was observed in the flow through, to obtain the purified **Man-NZ** solution. The concentration of **Man-NZ** was determined by measuring the absorption at 506 nm, and the **FeTPP** amount was determined by ICP−MS by tracking ^{56}Fe and ^{197}Au.

***Quantification of FeTPP/Man-NZ using ICP-MS*.** The quantification of Fe catalyst amounts per particle were performed by first adding 10 μL of **Fe-NZ** solution (10-30 μM) to a glass bottle containing 0.5 mL of H_2O_2/HNO_3 in a 1:1 ratio. The sample was dried overnight at 120 ^oC. Then, 0.5 mL of fresh aqua regia was added to provide the Fe/Au stock solution. The resulting solution was then diluted to 10 mL with de-ionized water. Sample composition was then analyzed on a Perkin-Elmer NexION 300X ICP mass spectrometer (located in the Institute of Applied Life Sciences). ^{197}Au and ^{56}Fe were measured under the standard mode: nebulizer flow rate, 0.95 L/min; rf power, 1600 W; plasma Ar flow rate, 18 L/min; dwell time, 50 ms.

Standard gold solutions (0, 20, 50, 100, 200, 250, 375 and 500 ppb) and iron solutions (0, 0.2, 0.5, 1, 2, 5, 10, 20, 30, 40, 45 and 50 ppb) were prepared via serial dilutions for the calibration curve.

Activation of pro-resorufin by Man-NZ. Each kinetic experiment was performed in a 96-well black plate with 100 μL of PBS solution containing the **Man-NZ** (500 nM), pro-resorufin[3] (**pro-res**, 20 μM), and 5 mM of glutathione. Samples were then immediately inserted into the plate reader at 37^{o}C to analyze the evolution of fluorescence (Ex. 568 nm, Em. 588 nm).

***Mammalian and bacterial cell culture*.** All mammalian cells were grown in T75 cell culture flasks using standard growth media [DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin (Pen-Strep)] for RAW 264.7 cells and EMEM supplemented with 10% FBS and 1% Pen-Strep for HEPG2 cells) under physiological conditions (37 °C in a humidified atmosphere of 5% CO_2). Once the cells were at sufficient density (~80% confluent), they were washed with phosphate-buffered saline (PBS) three times and detached using 0.05% trypsin. The cells were centrifuged and resuspended in fresh culture media. 10 μL of cell solution was mixed with trypan blue in a 1:1 ratio by volume and counted by hemocytometer. Cell solutions were diluted in the same media type to obtain the indicated concentrations for subsequent experiments.

Overnight cultures of bacteria were prepared by transferring an isolated colony from the designated agar plate to culture tubes with sterile LB broth for *Salmonella* and MRS broth for *Lactobacillus*. Bacterial cultures were then incubated overnight at 37 °C with agitation (275 rpm), until the stationary phase was reached. Bacteria were then collected by centrifugation (7000 rpm, 5 min) and washed thrice with 0.85% sodium chloride. Subsequently, the culture was resuspended in 1 mL of PBS to determine its OD_{600} (SpectraMax M2, Molecular Devices, San Jose, CA).

***NZ cellular uptake*.** RAW 264.7 and HEPG2 cells were seeded at concentrations of 50,000 cells/well in a 24-well plate overnight. The next day, the cells were treated with **Man-NZ/ COOH-NZ** at various concentrations for 24 h. The wells were washed three times with PBS and the cells were lysed by using 500uL of 1X Cell lysis buffer. The cell lysates were analyzed for gold content by ICP-MS.

For M2 phenotype of RAW 264.7 cells, 50,000 cells/well were stimulated by IL-4 (30 ng/mL) and seeded on a 24-well plate overnight, followed by indicated treatments.

***Evaluation of NZ retention by macrophages*.** RAW 264.7 cells were seeded at a concentration of 20,000 cells/well in three 24-well plates overnight. The next day, the cells were treated with **Man-NZ** at various concentrations for 24 h, 48 h and 72 h, respectively. The wells were washed three times with PBS and the cells were then lysed and further analyzed for gold content by ICP-MS.

Standard gold solutions were prepared via serial dilutions (0, 0.2, 0.5, 1, 2, 5, 10, and 20 ppb) for the calibration curve.

***Intracellular pro-fluorophore activation*.** RAW 264.7 cells were seeded at a concentration of 75,000 cells/well in a 4-well chamber Lab-Tek II chambered coverglass system 24 h before the experiment. The cells were washed with PBS and then incubated with 500 nM **Man-NZ** overnight. Media was then aspirated and the cells were washed with PBS to remove excess **Man-NZ**. 20 µM **pro-res** was then added in fresh media, and the cells were incubated for 2 h. The media was then replaced with PBS. Confocal microscopy images were obtained on a Zeiss LSM 510 Meta microscope by using a 40× objective. The settings of the confocal microscope were as follows: red channel: λex = 560 nm and λem = LP 640 nm. Emission filter: LP = high pass.

***Cytotoxicity*.** RAW 264.7 cells were seeded at a concentration of 10,000 cells/well in a 96 well plate overnight. The next day, the cells were treated with one of the following: **Man-NZ**, **pro-cip**, ciprofloxacin and **Man-NZ+pro-cip** at various concentrations for 24 h. After the incubation period, the cells were washed three times with PBS to remove dead cells and excess NZ. 10% Alamar Blue reagent (Invitrogen, Waltham, MA) in serum containing media was added to each well and incubated 2 h further at 37 °C and 5% CO_2. Cell viability was then determined by measuring the fluorescence intensity (ex/em: 560/590 nm) using a SpectraMax M5 microplate spectrophotometer. Three biological replicates were performed for viability determination.

Ninhydrin assay to determine pro-ciprofloxacin activation. Ninhydrin (Sigma Aldrich) assay was performed to demonstrate the activation of **pro-cip**, following the protocol provided by the vendor. 100 µL ciprofloxacin solutions of varying concentrations were prepared to generate a calibration curve. Then, 10 µL of 2% ninhydrin (in ethanol) solution was added to each **cip** solution and allowed to incubate for 10 min with shaking. Absorbance was then measured at 350 nm. 50 µL of **pro-cip** solutions of varying concentrations were mixed with 50 µL of 1000 nM **Man-NZ** to give a final concentration of 500 nM. Following this, the ninhydrin assay was performed as above and absorbance values were obtained. Concentration of activated **pro-cip** to **cip** was determined using the calibration curve.

***Bacteria killing assays*.** For the *Salmonella*-only infection model, a single colony of *Salmonella enterica* subsp*. enterica* serovar Typhimurium (ATCC 29630) was inoculated into LB broth and incubated at 37 °C overnight to reach stationary phase. Then 60 µL of the stationary phase solution was diluted to 3 mL using fresh LB broth and incubated at 37 °C until bacteria attained log phase growth. The resulting bacteria solution was then centrifuged, washed three times with 0.85% NaCl, and resuspended in PBS. Then, a solution containing $1x10^6$ CFU/mL *Salmonella* was prepared using LB broth. Subsequently, the test materials, 500 nM **Man-NZ**, 1 and 0.5 µM pro-ciprofloxacin, and 15 nM ciprofloxacin, were prepared using LB broth. 50 µL each of the bacteria and the test material were added to a 96-well microplate and incubated at 37 °C overnight with shaking. Quantitative colony counting was then performed to determine the numbers of bacteria that survived the treatment. Wells containing bacteria solution only served as the growth control while wells containing LB broth only served as the sterile control. Cultures were generated and analyzed in triplicates, and at least two identical, independent experiments were performed on different days.

For intracellular killing assays, a modified version of a previously published protocol was performed.[4] RAW 264.7 cells were seeded at a concentration of 17,000 cells/well in growth media lacking antibiotics with 500 nM **Man-NZ** in a 96-well plate, and allowed to grow overnight. *Salmonella* was then added to the wells to achieve 1:100 multiplicity of infection (MOI) and incubated for 1 h. The wells were washed with gentamicin three times and either pro-ciprofloxacin or ciprofloxacin was added in fresh media, then incubated overnight. The following day, the media was removed, the mammalian cells were lysed using 0.01% Triton X, and the lysate was collected for quantitative colony counting. Three biological replicates were generated and analyzed for each group.

Transwell membrane bacterial co-culture assays. To model the selective killing of intracellular macrophage pathogens over non-pathogenic gut bacteria by **Man-NZ+pro-ciprofloxacin**, a transwell membrane assay was developed. The probiotic Bio-K^+ (*Lactobacillus* sp.) was used as a model of the intestinal flora. Briefly, RAW 264.7 cells were seeded overnight in a 6-well plate in DMEM with 10% bovine calf serum and 500 nM **Man-NZ** at 37 °C. Intracellular *Salmonella* infection was performed using the same procedure described above. On the other hand, Bio-K^+ was cultured in MRS broth overnight at 37 °C with shaking to reach stationary phase. Then, a solution of Bio-K^+ was prepared using 1:1 MRS:DMEM media and added to a transwell membrane (0.4 µm pore size), which was inserted into the 6-well plate containing infected macrophages. Subsequently, the test materials were prepared using 1:1 MRS:DMEM media and added to each of the wells. The plate was incubated overnight at 37 °C. The media was then collected and used for quantitative colony counting using MRS agar to determine numbers of surviving *Lactobacillus* sp. The infected macrophages were washed using PBS and lysed with 0.01% Triton X. The lysate was recovered and plated in Xylose Lysine Deoxycholate (XLD) agar to quantitatively determine surviving numbers of *Salmonella*.

Statistical analysis. For all bacteria and macrophage viability experiments, data are presented as mean ± standard deviation of at least three replicates. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A P value < 0.05 is considered as statistically significant. GraphPad Prism was used to perform statistical analyses.

Supplementary figures

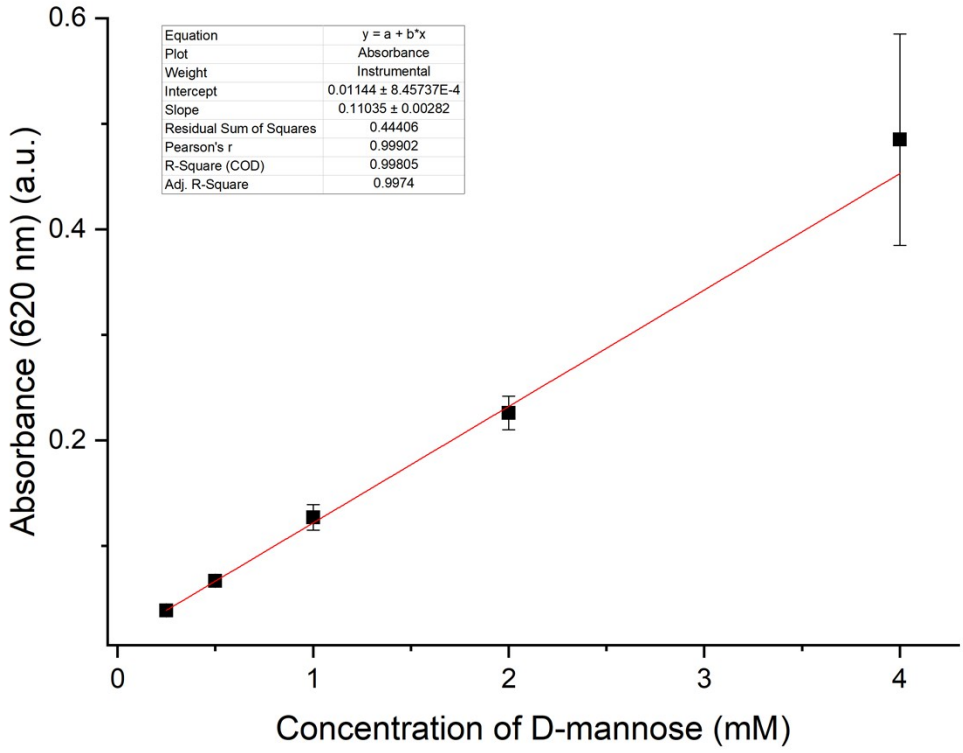


Figure S1. Calibration curve obtained from treating various concentrations of D-mannose with anthrone/sulfuric acid and measuring the absorption at 620 nm.

Calculation of D-mannose density on AuNP.

The maximum number of D-mannose molecules on each Au NP is calculated as follows:

D-mannose-functionalized AuNP (**Man-AuNP**) concentration: 5 μM

No. of moles of AuNP = M x V = 5 μM x 0.5 mL = 2.5 nmol

No. of AuNPs = 2.5 nmol x 6.02 x 10^{23} = 1.5 x 10^{15}

1 AuNP has ~70 ligands (previously described)[5,6]

Therefore, 1.5 x 10^{15} AuNPs have 1.5 x 10^{15} x 70 = 1.05 x 10^{17} ligands

If 100% coverage by D-mannose is obtained after EDC/NHS coupling on COOH-AuNP, then 1.05 x 10^{17} mannose units will be present on AuNP, therefore,
Theoretical no. of D-mannose = 1.05 x 10^{17}

By Anthrone-sulfuric acid assay, concentration of D-mannose in 5 μM AuNP = 0.139 mM

No. of moles of D-mannose = M x V = 0.139 mM x 0.5 mL= 69.5 nmol

No. of D-mannose molecules (or Experimental no. of D-mannose) = 69.5 nmol x 6.02 x 10^{23} = 4.18 x 10^{16}

Therefore, % of D-mannose on **Man-AuNP** = {[Experimental no. of D-mannose]/[Theoretical no. of D-mannose]} x 100 = [4.18 x 10^{16}]/[1.05 x 10^{17}] x 100 = 39.84 % = **~40%**

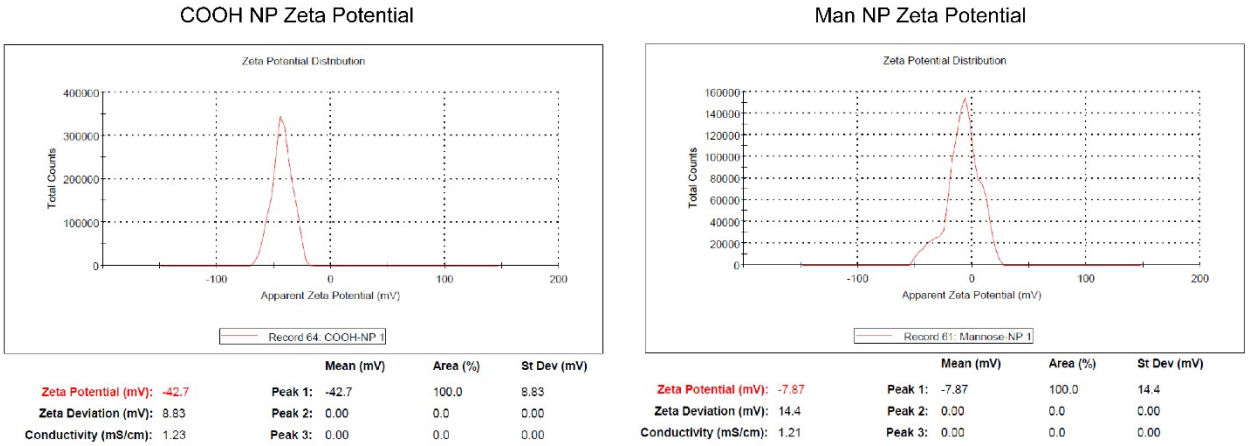
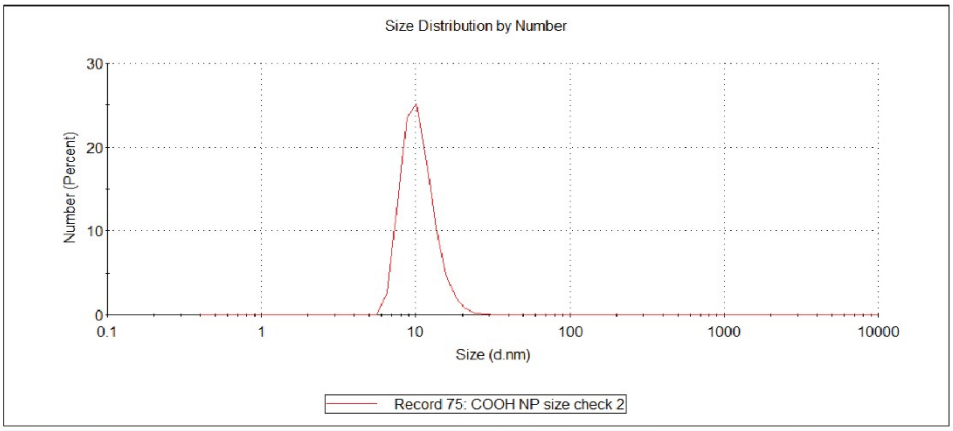


Figure S2. Zeta potential of AuNPs before (left) and after (right) functionalization with mannose.

		Size (d.nm):	% Number:	St Dev (d.nm):
Z-Average (d.nm): 22.99	Peak 1:	10.56	100.0	2.730
PdI: 0.538	Peak 2:	0.000	0.0	0.000
Intercept: 0.718	Peak 3:	0.000	0.0	0.000

Result quality : Good



		Size (d.nm):	% Number:	St Dev (d.nm):
Z-Average (d.nm): 44.91	Peak 1:	12.83	100.0	3.200
PdI: 0.928	Peak 2:	0.000	0.0	0.000
Intercept: 0.774	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report

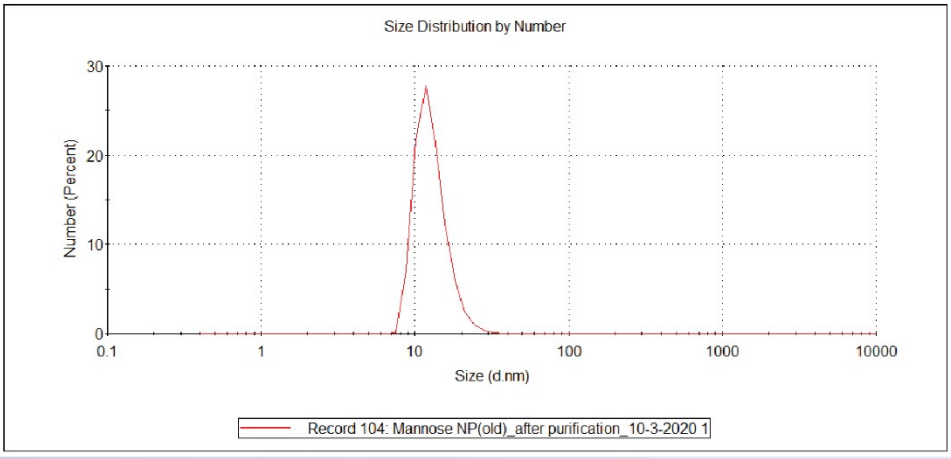


Figure S3. Size determination of AuNPs before (top) and after (bottom) functionalization with mannose.

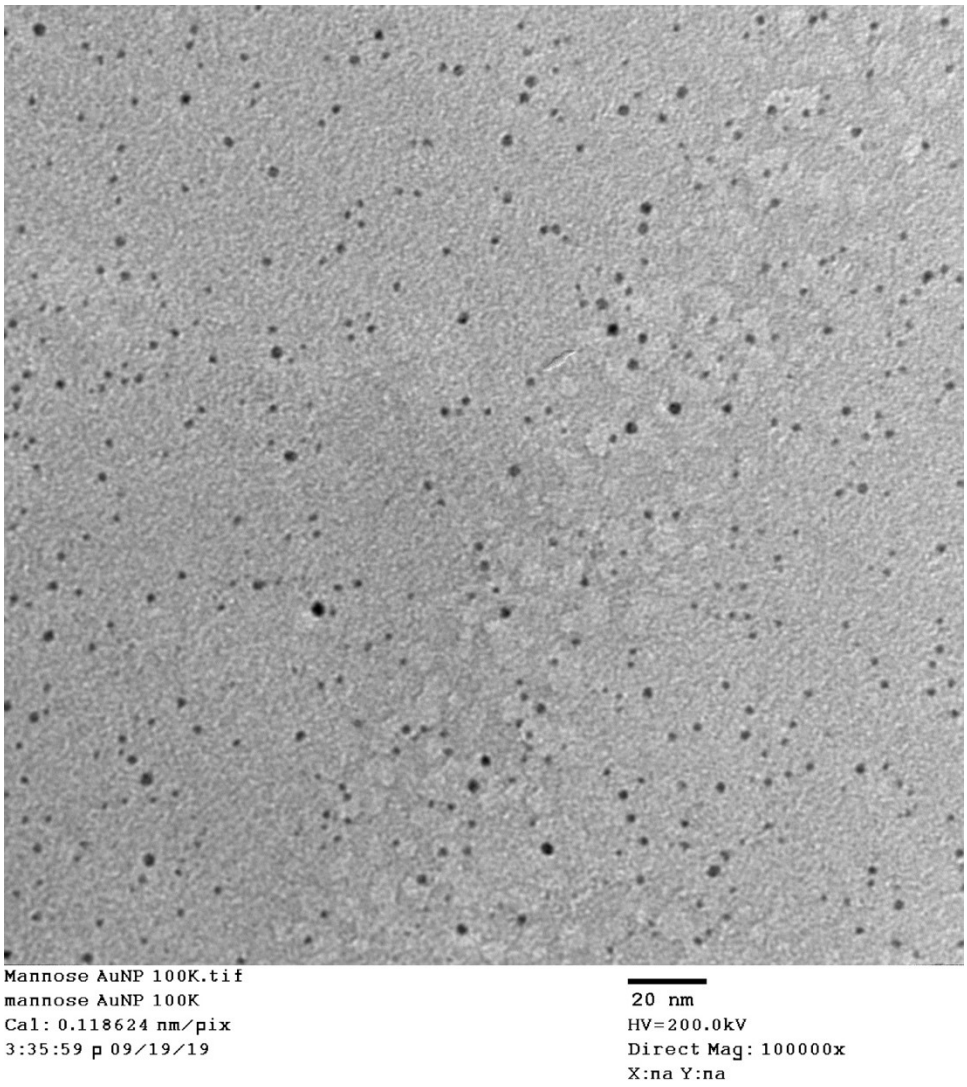


Figure S4. TEM image of **Man-NZ**. Scale bar represents 20 nm.

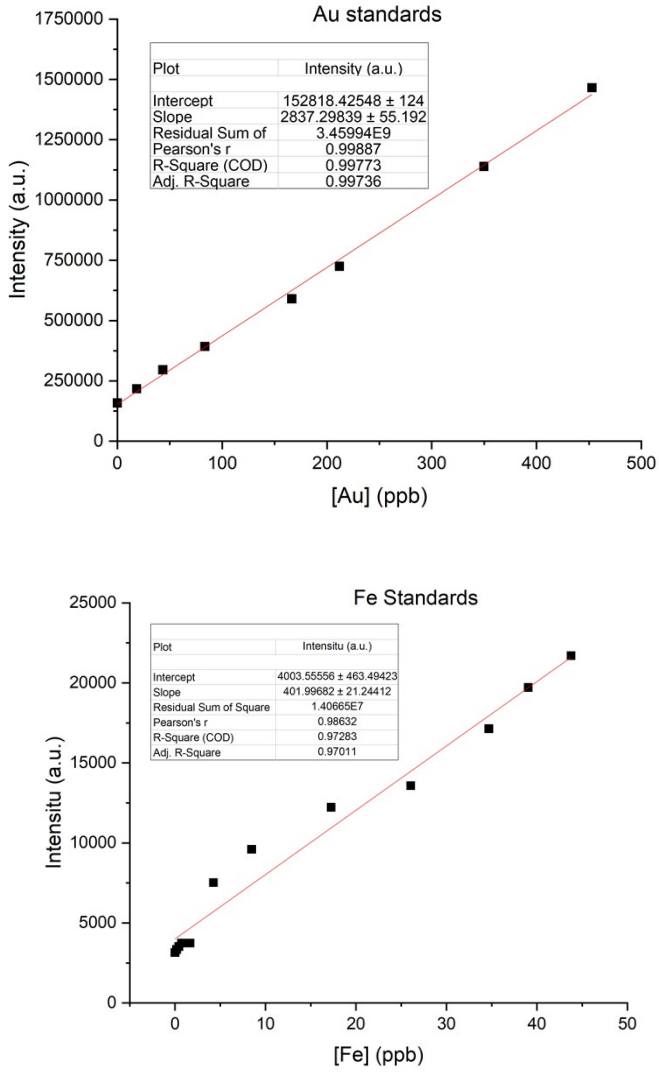


Figure S5. Standard calibration curves for Au (top) and Fe (bottom).

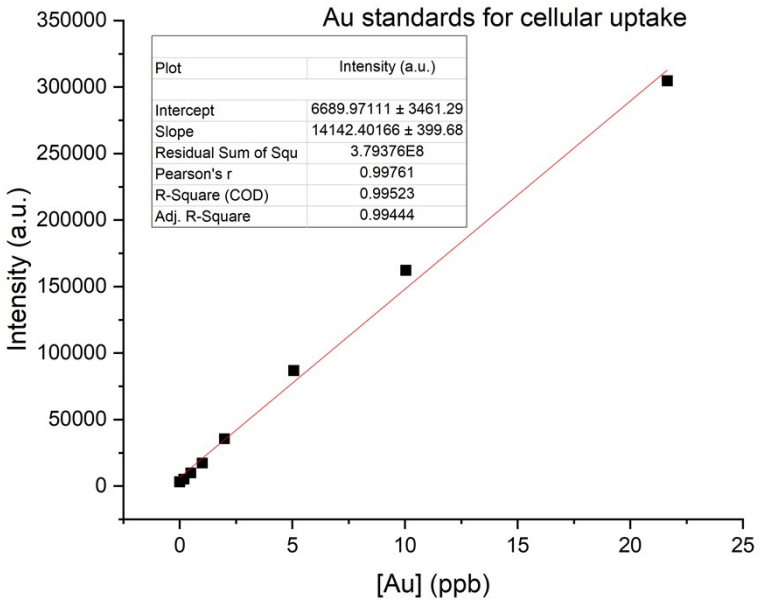


Figure S6. Au calibration curve for **Man-NZ** uptake by macrophages.

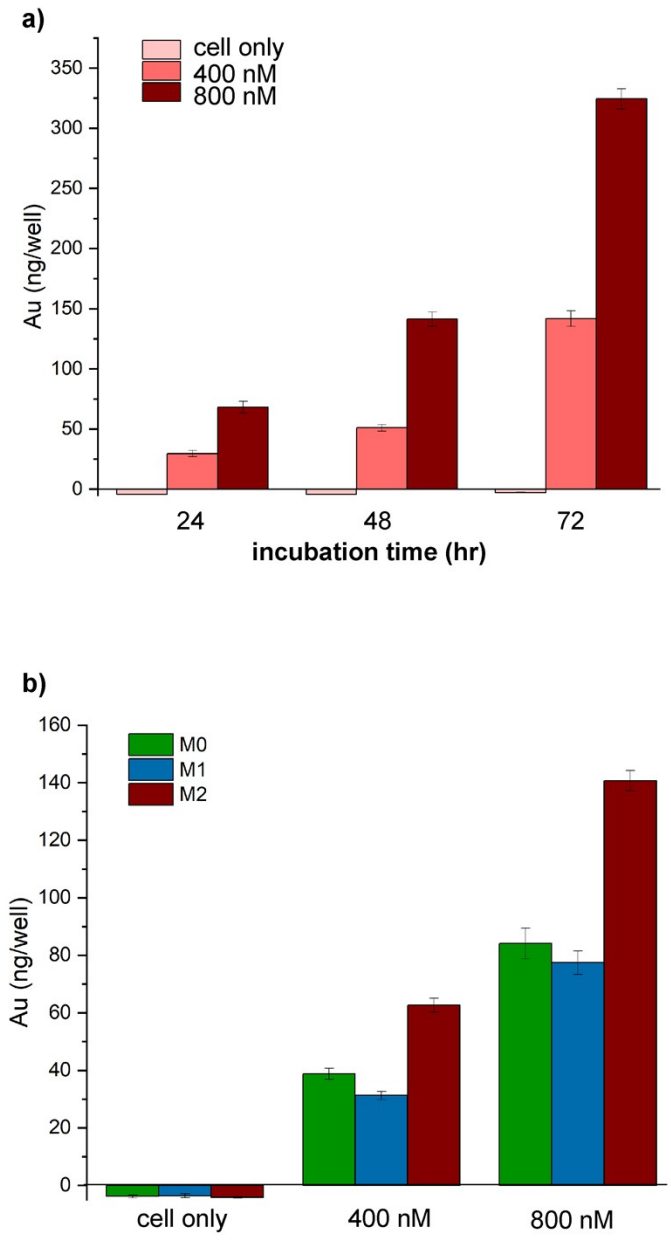


Figure S7. ICP-MS analysis of gold accumulation in a) RAW 264.7 macrophages following 24, 48, and 72 h incubation with **Man-NZ** and b) M1 and M2 polarized macrophages after 24h **Man-NZ** incubation. Error bars represent standard deviation (n=3).

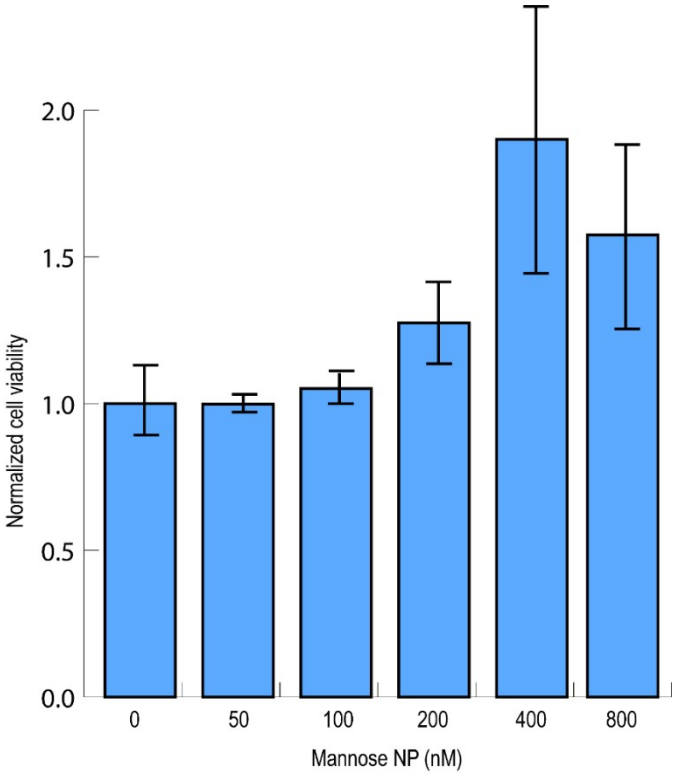


Figure S8. Cytotoxicity of **Man-NZ** to RAW 264.7 macrophages. Data are presented as mean ± standard deviation, n=3. All treatment groups have no statistically significant difference from the control group.

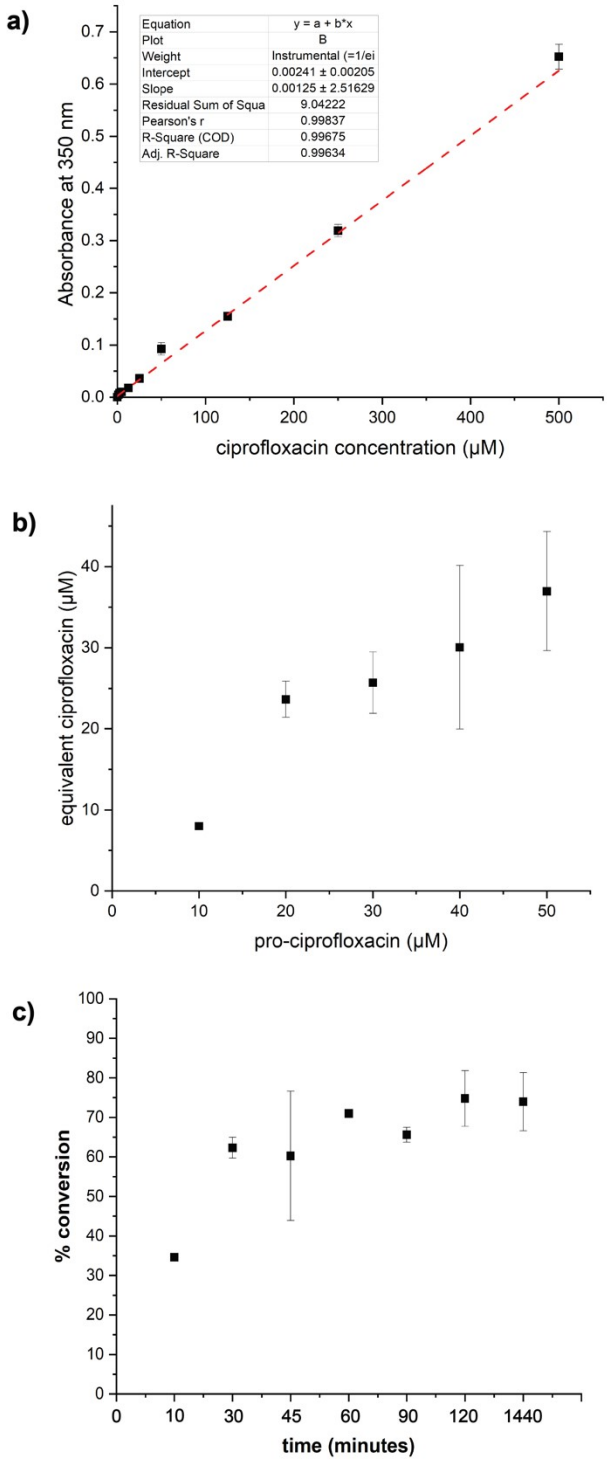


Figure S9. Validation of **pro-cip** conversion to ciprofloxacin using Ninhydrin assay. a) Calibration curve for Ninhydrin assay. b) Equivalent ciprofloxacin concentration from activated pro-drug. c) Percent conversion of 50 µM **pro-cip** with 500 nM **Man-NZ** over time. Data are presented as mean ± standard deviation, n=3.

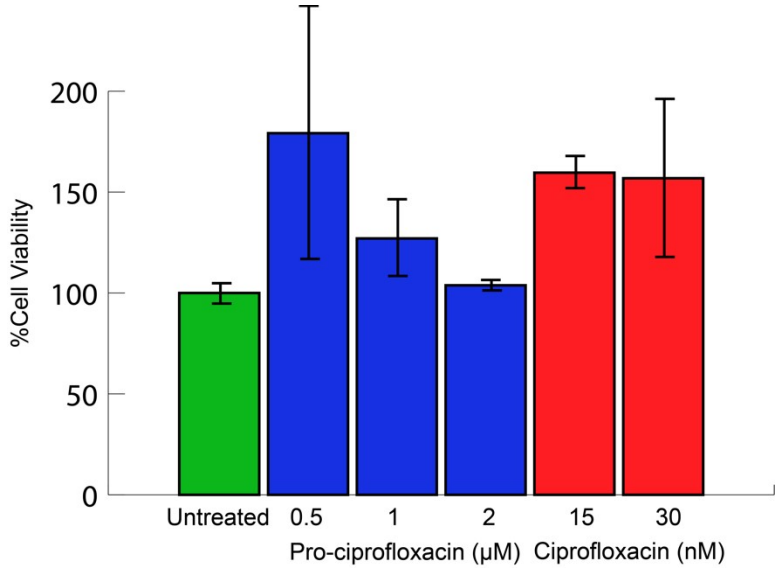


Figure S10. Cytotoxicity of pro-ciprofloxacin and ciprofloxacin to RAW 264.7 macrophages. Data are presented as mean ± standard deviation, n=3.

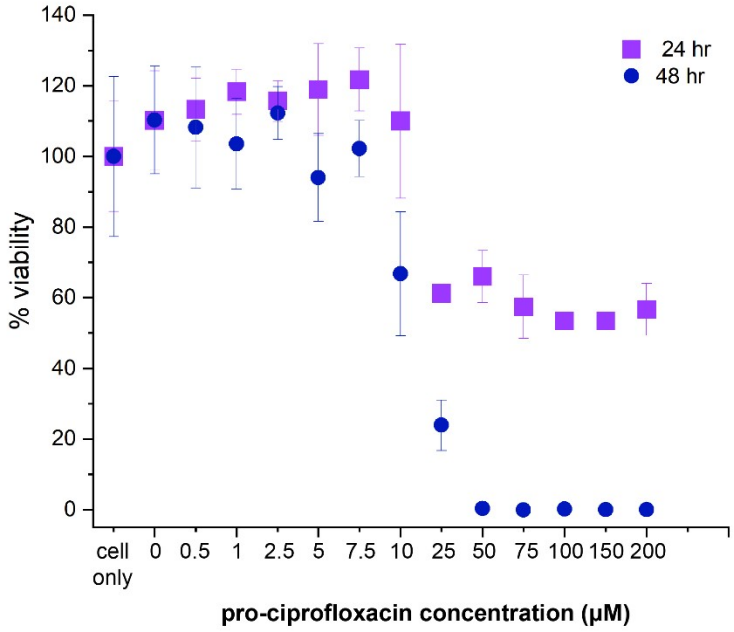


Figure S11. Cytotoxicity of **Man-NZ+pro-cip** in RAW 264.7 macrophages. Increasing pro-ciprofloxacin concentration was used while **Man-NZ** was kept constant at 500 nM. Viability was monitored by Alamar Blue assay after 24- and 48-hours of incubation. Data are presented as mean ± standard deviation, n=3.

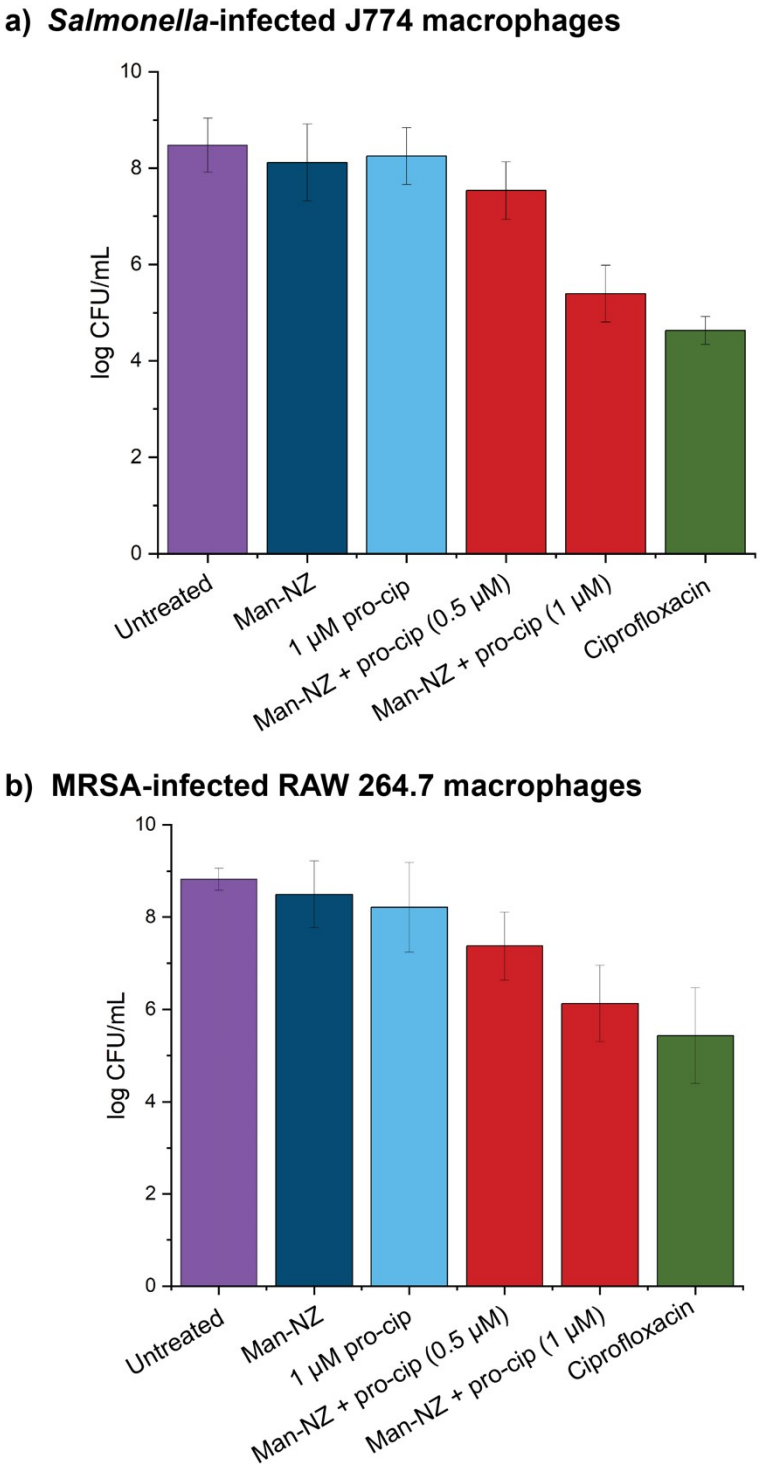


Figure S12. Activity of **Man-NZ**+pro-cip against different intracellular infection models. a) Viability of *Salmonella* residing inside J774 a.1 macrophages after 24 hr treatment with **Man-NZ**+**pro-cip**. b) Viability of MRSA residing inside RAW 264.7 macrophages after 24 hr treatment with **Man-NZ+pro-cip** quantified by colony counting. Data are presented as mean ± standard deviation, n=3.

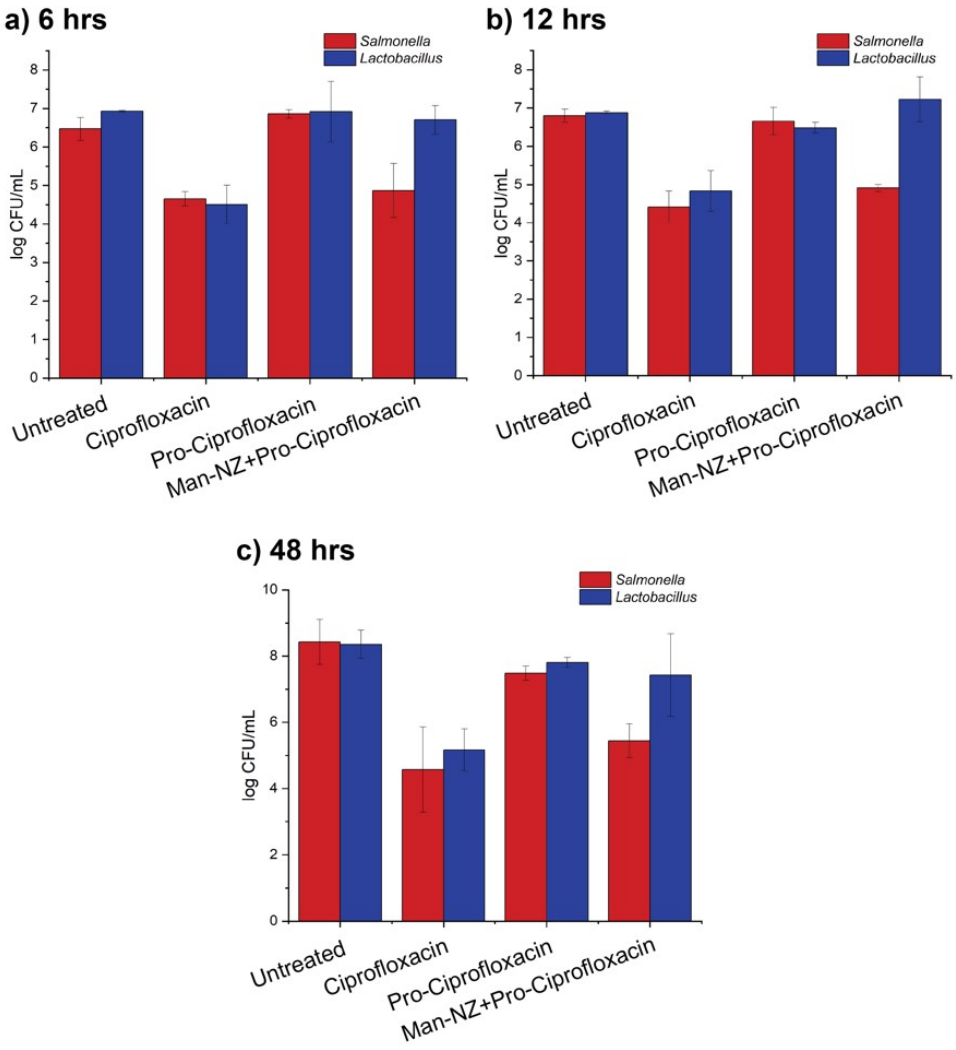


Figure S13. Viability of *Lactobacillus* sp. and intracellular *Salmonella* after 6-, 12- and 48 h treatment with **Man-NZ+pro-cip** determined by quantitative colony counting. Data are presented as mean ± standard deviation, n=3.

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