Supporting Information for

Erythrocyte-mediated delivery of bioorthogonal nanozymes for selective targeting of bacterial infections

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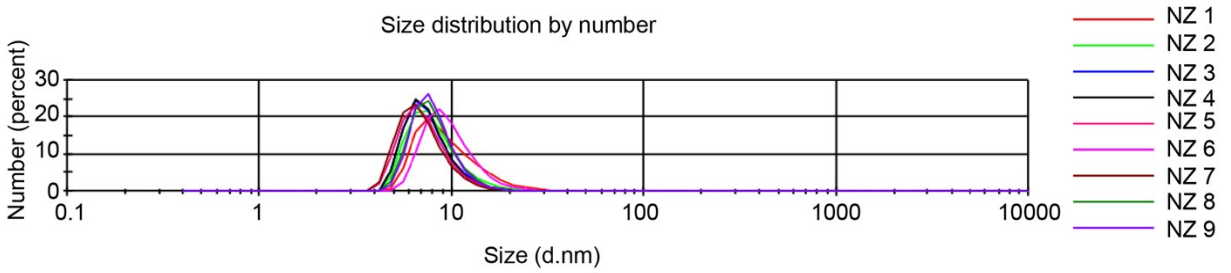
Synthesis of gold nanoparticles (AuNPs). The ligands were synthesized according to the previously published papers.[1,2,3,4] Briefly, 2 nm AuNPs capped by 1-pentanethiol were synthesized following Brust-Schiffrin method. The thiol ligands were dissolved with the AuNPs to perform place exchange reaction in dichloromethane (DCM) for 3 h in room temperature under argon atmosphere. The solvent was removed followed by dissolving the precipitate in distilled water. The excess ligands were removed by dialysis using a membrane (molecular cut-off = 10,000). AuNPs were lyophilized and were redispersed in deionised water.

Catalyst encapsulation in the NP monolayer (nanozymes). Equal volumes of catalyst ([Fe(TPP)]Cl 5,10,15,20-tetraphenyl-21H,23H-porphine (TPP)-containing complex, ~3mM) and AuNPs (10 μM, 0.5 ml) were dissolved in an mixture of water/THF, followed by slow evaporation of THF. Slow evaporation of organic solvent (THF) enabled immobilization of catalysts in the NP monolayer, generating nanozymes (NZs). Excess catalysts precipitated in the solution and were filtered out using Millex-GP filter (25 mm PES, pore size: 0.22 μm) and dialysis (Snake Skin dialysis tubing, 10K) against water (5 L) for 24 h. NZs were further purified to remove free catalysts through multiple filtrations using Amicon ultra 4 centrifuging tubes (10K) remove free catalysts.

DLS characterization of nanozymes. The hydrodynamic diameter of the nanozymes were measured by dynamic light scattering (DLS) in DI water using Malvern Zetasizer Nano ZS instrument. The measurement setting were as follows: angle: 173° (backscatter), “multiple narrow modes” (high resolution) based on non-negative-least-squares (NNLS).

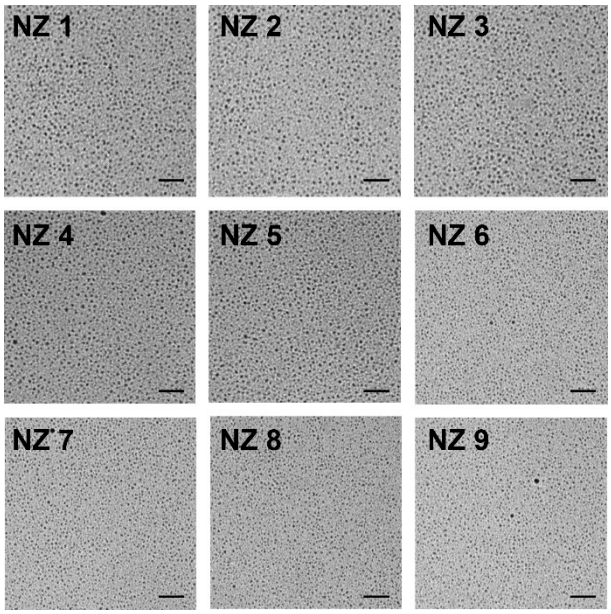


SI Figure S1. DLS measurements of NZ 1 - NZ 9.

Nanozyme	Hydrodynamic diameter (nm)
NZ1	9.1 ± 2.1
NZ2	9.8 ± 2.3
NZ3	8.7 ± 3.5
NZ4	8.7 ± 0.9
NZ5	8.1 ± 1.9
NZ6	8.1 ± 2.4
NZ7	8.1 ± 3.7
NZ8	8.4 ± 4.1
NZ9	9.4 ± 2.9

Supplementary Table S1. showing the NZ size, zeta potential, hydrophobicity indices of functional group, number of NZs per RBC and hemolytic effect caused by NZs at 500 nM concentration to RBCs

TEM characterization of nanozymes. Transmission Electron Microscopy (TEM) images of samples were taken using JEOL CX-100 electron microscopy. Samples were prepared by placing one drop of the desired solution onto a 300-mesh Cu grid-coated with carbon film. No aggregation was observed after catalyst encapsulation.



SI Figure S2. TEM images of nanozymes. Scale bar = 20 nm.

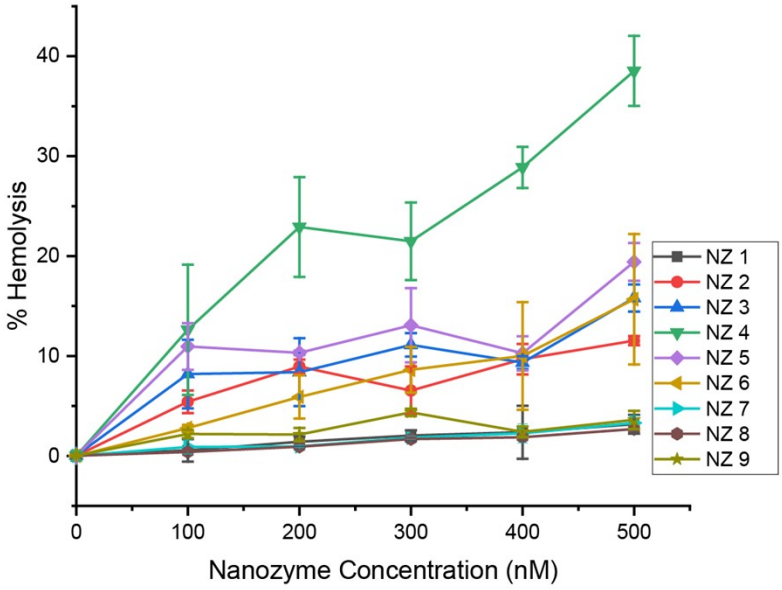
Quantification of catalyst per AuNP using ICP-MS characterization. The catalysts were quantified using previously established protocols.[5] Briefly, the nanoparticle solutions were diluted in milli-Q water to 200 nM. 10 μL of the diluted solution was taken for each replicate. 0.5 mL of aqua regia was added to each replicate and was diluted to 10 mL by adding milli-Q water. The amount of encapsulated catalysts was measured by a Perkin-Elmer NexION 300X ICP mass spectrometer by tracking ^{56}Fe relative to ^{197}Au. For calibration, a series of solutions with gold and iron (concentration: 0, 0.2, 0.5,1, 2, 5, 10, and 20 ppb) were prepared. Operating conditions were as follows: nebulizer flow rate: 0.95 L/min; rf power: 1600 W; plasma Ar flow rate: 18 L/min; dwell time: 50 ms.

Nanozymes	Au(ppb)	Fe(ppb)	Fe/AuNP
1	24.6	1.2	35.6 ± 2.1
2	27.7	1.3	33.2 ± 1.1
3	27.3	1.1	34.8 ± 5.9
4	24.6	0.9	34.9 ± 3.6
5	27.1	1.4	33.1 ± 1.8
6	25.9	1.1	35.4 ± 7.1
7	26.6	1.2	30.2 ± 10.3
8	22.9	1.0	30.7 ± 9.9
9	28.7	1.2	31.1 ± 8.2

Supplementary Table 2. Gold (Au) and iron (Fe) amount in the nanozymes using ICP-MS measurement. The Fe/AuNP represents number of catalysts encapsulated per AuNP.

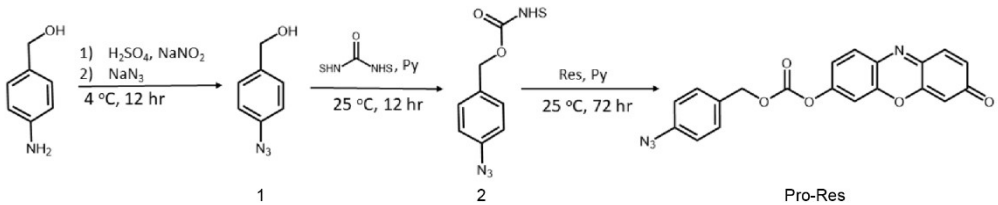
Hemolysis assays with nanozymes. Hemolysis assays on red blood cells were performed using previously reported protocols.[6] Briefly, human whole blood (pooled, mixed gender) was purchased from Bioreclamation LLC, NY and processed as soon as received. 10 ml phosphate buffered saline (PBS) was added to the blood and centrifuged for 5 minutes at 5000 r.p.m. Supernatant was discarded followed by redispersion of RBCs in 10 ml of PBS. This cycle was repeated at least 5 times. Finally, RBCs were diluted in 10 ml of PBS and kept on ice during sample preparation. 0.4 ml of NZs (different concentrations) were prepared in a 1.5 ml Eppendorf tube, followed by addition of 0.1 ml of RBC solution. RBCs incubated with water and PBS were used as negative and positive controls respectively and all the samples were prepared in triplicates. These mixtures were incubated at 37 °C, 150 r.p.m for 24 hours. The mixtures were then centrifuged at 4000 r.p.m for 5 minutes and 100 µl of supernatant was transferred to a 96-well plate. The absorbance of supernatant was measured at 570 nm using Molecular Devices SpectraMax M2 microplate reader with 655 nm as a reference.

Nanozyme adsorption on RBCs. RBCs were isolated from human whole blood as described in the above section. Next, nanozymes (NZ 1-9) with varied concentrations (100 – 1000 nM) were incubated with RBCs in PBS for 1 hour at 37 °C, 150 r.p.m. The NZ-RBC mixture were then washed with PBS five times at 4000 r.p.m for 5 minutes to remove excess NZs in the solution. The isolated RBCs were then processed for ICP-MS analysis and quantified for gold content.



SI Figure S3. Dose-dependent hemolytic activity of NZ 1–NZ 9 in the presence of plasma proteins. % hemolysis was calculated using water as the positive control. Error bars represent standard deviations (n = 3).

Synthesis of aryl-azide protected resorufin substrate (pro-res).



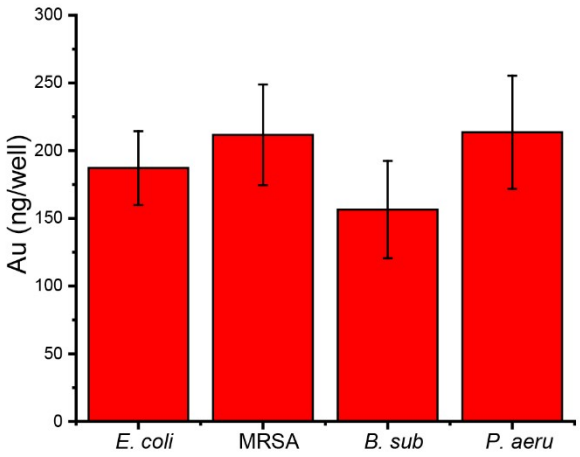
Compound 1 was synthesized from the corresponding aminated compound. A solution of 4-aminobenzyl alcohol (2.15 g, 17.5 mmol) was dissolved in THF (25 mL) and mixed with an aqueous solution of H_2SO_4 (4.8 mL 98%, in 60 mL of water) cooled at 4 ^oC in a round bottom flask. An aqueous solution of $NaNO_2$ (1.45 g, 21 mmol) was added to the solution and was allowed to react for 1 h in ice bath. An aqueous solution of NaN_3 (1.8 g, 28.9 mmol) was added to the solution. The mixture was led to react overnight followed by collecting 4-azidobenzyl alcohol by three liquid-liquid extraction processes using dichloromethane (100 mL). Fractions were collected and dried over anhydrous Na_2SO_4, concentrated and purified by flash column chromatography (DCM: MetOH 19:1). Yield 84%.

Nanozyme kinetics in solution. Pro-res (non-fluorescent) was used as a substrate to test the catalytic activity of the nanozymes. A solution containing 10 μM substrate and RBC-NZs (100 – 1000 nM) was prepared in a 96-well plate. A solution of 500 nM free nanozyme (Bare-NZ) with 10 μM substrate was used for comparison. Solutions of RBC-NZs, 10 μM substrate, RBCs alone were used as negative controls. The kinetic study was done by tracking the fluorescence intensity (ex = 561 nm, em = 590 nm) using a Molecular Devices SpectraMax M2 microplate reader.

Biofilm Culture. Biofilms were cultured as reported in previously established protocols. Briefly, bacteria were grown overnight in LB media at 37 ˚C until to reach stationary phase. Bacteria cultures were then centrifuged and washed three times using 0.85% sodium chloride solution. Bacteria were resuspended in PBS and their concentrations were determined using optical density measurements at 600 nm. Seeding solutions were made in M9 media to reach an OD_{600} of 0.1. 500 μL of the seeding solutions were added to each well of the 12-well microplate (100 μl seeding soltions were used for 96-well plates). The plates were covered and incubated at room temperature for 24 hours in static conditions.

Hemolytic activity of bacterial strains. Biofilms were cultured in a 12-well plate using the above-mentioned protocol. Red Blood Cells were isolated from human whole blood and suspended in PBS. 500 µl of RBC solution was added to the 12-well plates containing biofilms and incubated at 37 °C, 150 rpm for 30 minutes and 24 hours. RBC solution incubated in PBS and milli-q water (500 µl) were used positive and negative controls respectively. Solutions from 12-well plate were collected in a 1.5 ml eppendorf tubes and centrifuged at 4000 rpm for 5 minutes. The supernatant was transferred to a clear 96-well microplate and absorbance was measured at 570 nm using Molecular Devices SpectraMax M2 microplate reader with reference at 655 nm.

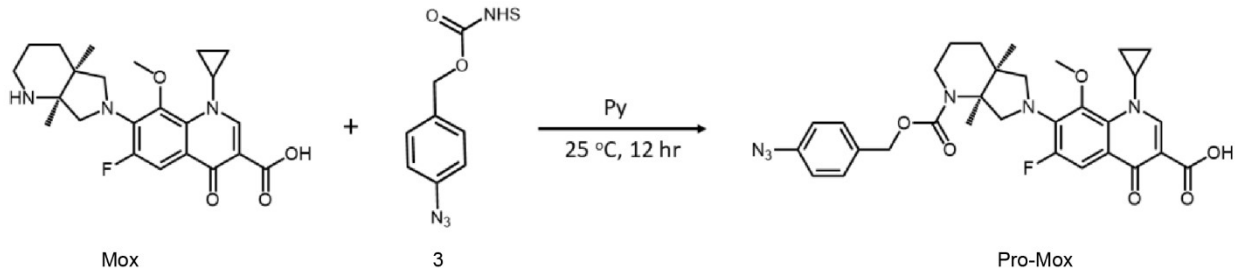
Nanozyme accumulation studies in biofilms. 0.1 OD_{600} bacterial cells were plated in a 12-well plate for 24 hours. On the following day, media was replaced with fresh M9 minimal media and further incubated for 24 hours. Biofilms were then washed three times with PBS, followed by incubation with RBC-NZs (500 nM) in minimal M9 media for 24 hours at 37 °C. Biofilms incubated with free NZs (Bare-NZs) and media alone were used as controls. After incubation, the biofilms were washed with PBS three times, followed by addition of lysis buffer to each well. All lysed samples were then further processed for ICP-MS analysis (*vide infra*) to determine the intrabiofilm amount of gold. Nanozyme accumulation experiments were performed at least two times, and each experiment comprised three replicates.



SI Figure S4. Amount of gold (Au) accumulated in biofilms obtained from ICP-MS upon treatment with free NZ 1.

Confocal imaging of biofilms. 10^8 cfu/ml of GFP expressing methicillin-resistant *S. aureus* supplemented with 1mM of IPTG (isopropyl β-D-1-thiogalactopyranoside) bacteria solution (2 ml in M9 media) was seeded in a confocal dish and allowed to grow for 3 days. Old media was replaced every 24 hours. After 3 days, media was replaced by RBC-NZs (500 nM) and biofilms were incubated for 3 hours. Biofilms were then washed with PBS three times to remove excess NZs or RBC debris from the solution, followed by addition of 20 μM Pro-Res (substrate) for 1 hour. The biofilms were then washed three times with PBS and visualized under a confocal microscope. Confocal images were obtained on a Zeiss LSM 519 Meta microscope by using a 63× objective. The settings of the confocal microscope were as follows: green channel: λ_{ex}=488 nm and λ_{em}=BP 505-530 nm; red channel: λ_{ex}=543 nm and λ_{em}=LP 650 nm. Emission filters: BP=band pass, LP=high pass.

Synthesis of aryl-azide protected moxofloxacin substrate (pro-Mox).



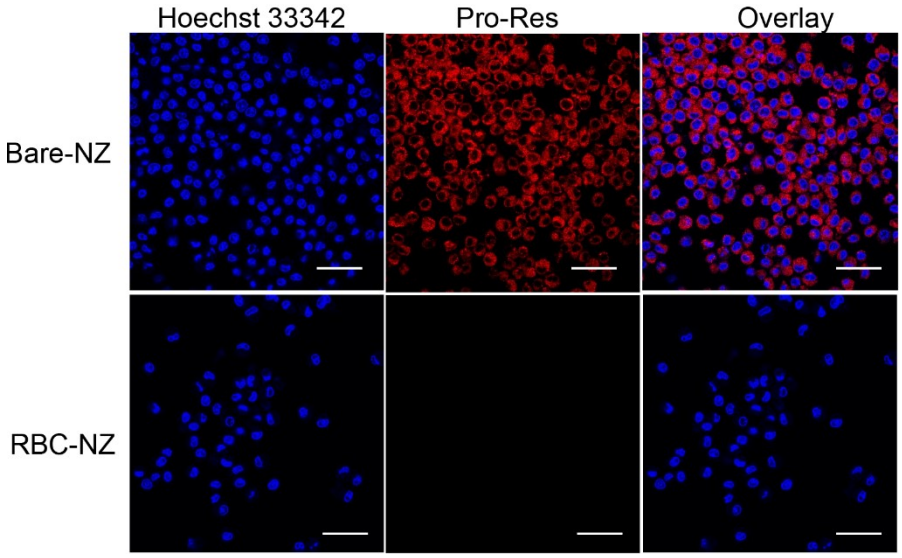
Mox 3 Pro-Mox

The compound 3 (100 mg, 1 eq) was added to a rounded bottom flask containing a solution of Mox (100 mg, 0.75 eq) and 50 mg of solid $NaHCO_3$ (1.2 eq) in anhydrous DMF (5 mL). The mixture was left to react overnight. Cold water was added and the product was extracted by washing with ethyl acetate. All organic fractions were then collected and dried over anhydrous Na_2SO_4, concentrated and purified by recrystallization in ethyl acetate. Yield 64 %.

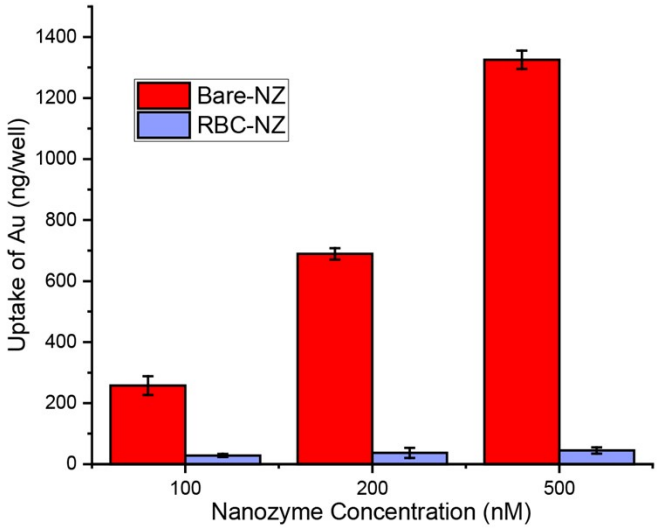
Cellular uptake experiments. 20,000 RAW 264.7 macrophage cell lines (purchased from American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM; ATCC 30-2002) in the presence of 10% bovine calf serum and 1% antibiotic solution at 37 ºC in a humidified atmosphere of 5% CO_2 for 48 h. Old medium was replaced and washed with PBS. Meanwhile, RBC-NZs (100, 250 and 500 nM) solutions were prepared in prewarmed 10% serum containing media. Next, the cells were incubated with RBC-NZs (100, 250 and 500 nM) in 10% serum-containing media for 24 h at 37 °C. Free nanozymes (Bare-NZs) were used as controls. Subsequently, the cells were washed three times with PBS and treated with lysis buffer. Lysed cells were then processed for ICP-MS analysis. Each experiment comprised of 3 replicates.

Confocal imaging of macrophage cells. Macrophages were seeded in a confocal disc at a concentration of 200,000 cells/mL in a 10% serum containing media. After 24 h, the cells were

treated with RBC-NZ and were stored in incubator for overnight. Next, the cells were washed three times with PBS and were incubated with pro-resorufin for 0.5 h. Hoechst 33342 was used as a nuclear staining dye. The cells treated with free NZ and proflurophore only were used as positive and negative controls respectively.

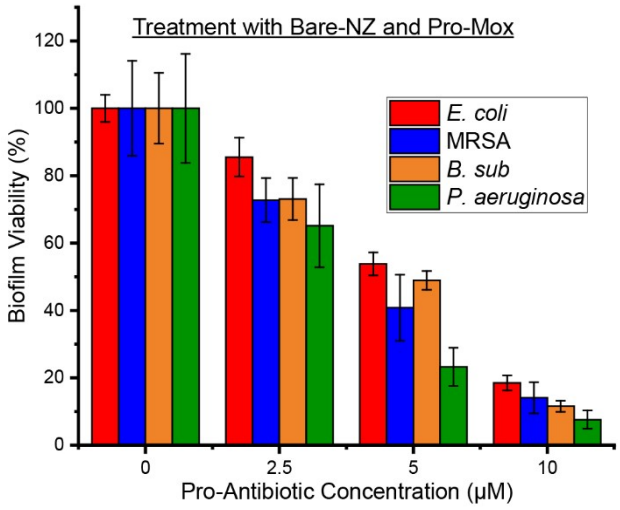


SI Figure S5. Confocal images of macrophage cells (RAW 264.7) incubated with RBC-NZ and Bare-NZ in presence of pro-resorufin. No fluorescence was generated by RBC-NZ, indicating that the RBC-hichhiked nanozymes were not uptaken by macrophages. Hoechst 33342 was used as a nuclear staining dye. The scale bars are 25 μm.

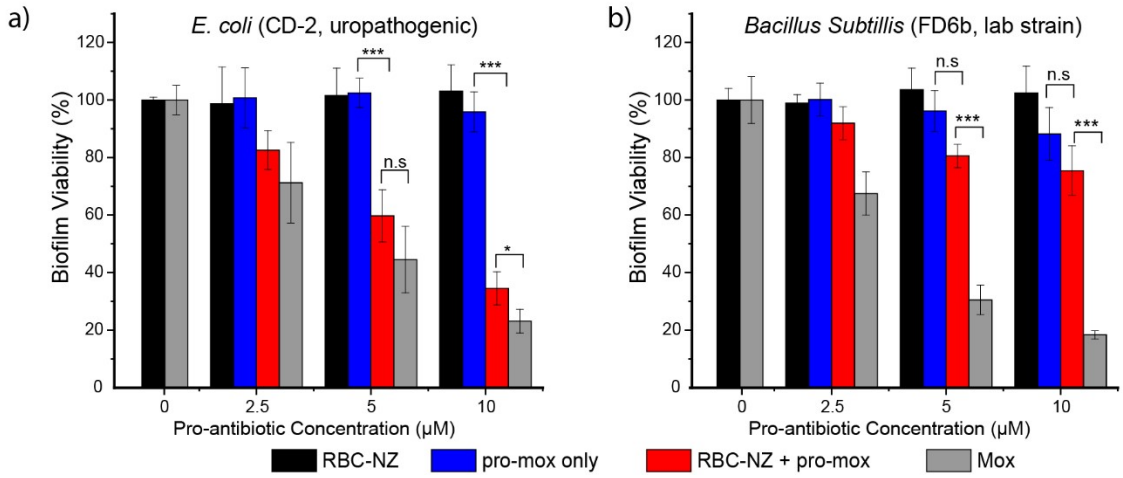


SI Figure S6. Nanoparticle uptake by RAW 264.7 macrophage cells biofilms after incubation for 24 h in pH 7.4 media with Bare-NZ and RBC-NZ (500 nM), as measured by ICP-MS. These results indicate that RBC hitchhiking prevents uptake of NZ 1.

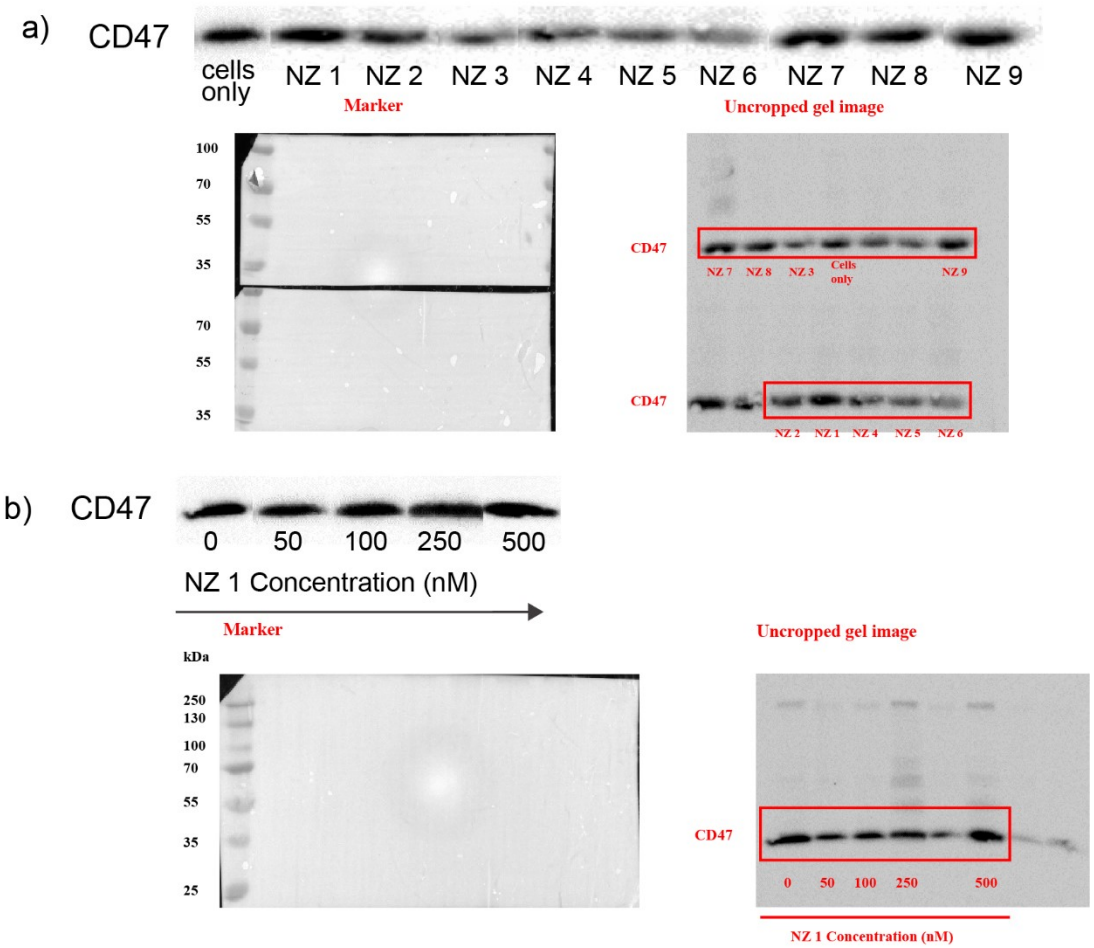
Biofilm treatment using pro-antibiotic activation. 10^8 cfu/ml of bacteria seeding solution was prepared in minimal M9 media. 100 µl of the seeding solution was added to each well of the 96-well microplate. The plates were covered and incubated at room temperature for 24 hours at static conditions. On the following day, solution of RBC-NZs was prepared in M9 media and diluted to desired levels and incubated with biofilms for 24 hours at 37 °C overnight without shaking. Biofilms were then washed with PBS to remove unattached NZs or RBC debris, and incubated with pro-mox (1-10 µM) diluted in M9 minimal media for 24 hours at 37 °C. The cells were then washed three times with PBS and incubated with 10% alamar blue in minimal M9 media for 3 hours at 37 °C. Biofilm viability was then determined by measuring fluorescence intensity at 570 nm using a Molecular Devices SpectraMax M2 microplate reader. Biofilms incubated with RBC-NZs, pro-mox and moxifloxacin only were used as controls.



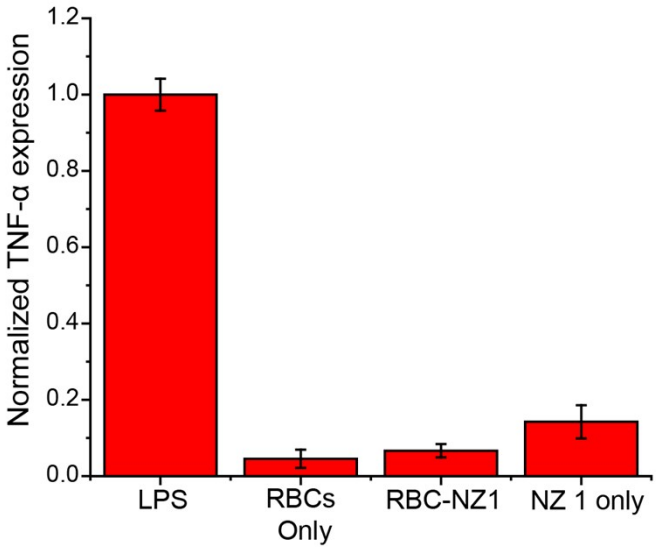
SI Figure S7. Viability % of biofilm treated with bare-NZ with increasing concentration of pro-Mox.



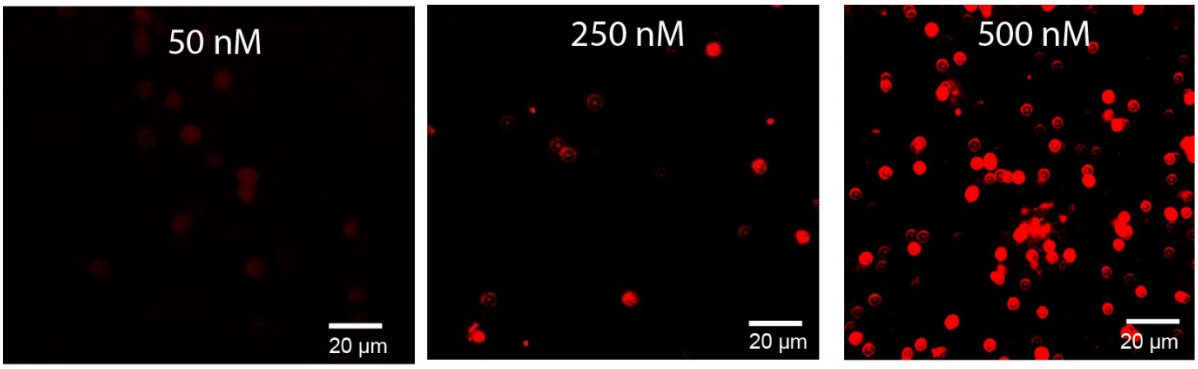
SI Figure S8. Deprotection of antimicrobials in biofilms using RBC-hitchhiked nanozymes. **a.** *E. coli* (toxin producing) biofilms and **b.** *B. Sub* (non-pathogenic) biofilms treated with pro-Mox and RBC-NZ (red bars) at 37 °C. Biofilms treated only with pro-Mox (blue bars) or with Mox (grey bars) were used in all experiments as negative and positive controls, respectively. Each experiment was replicated five times. Error bars represent standard deviations of these measurements. *$p <$ 0.05, ***$p < 0.001$



SI Figure S9. Red blood cell membrane protein CD47 was monitored in RBCs hitchhiked with a) NZ 1-9 and compared with freshly derived RBCs as controls. Each sample was prepared using same number of cells and nanozymes for western blotting. It was observed that NZ 1,2,7,8,9 did not alter CD47 expression on RBCs. b) Different concentrations of NZ 1 were tested to determine their effect on CD47 expression in RBCs. No Significant difference in CD47 expression was observed for upto 500 nM of NZ1. The lanes have been cropped and rearranged in the figure for comparative purposes.



SI Figure S10. TNF-α secretion of Raw 264.7 cells in the presence of RBC-NZ1, RBCs alone and Bare-NZ1 (non-hitchhiked NZ1). Lipopolysaccharide (LPS) was used as a positive control.



SI Figure S11. Confocal images of RBCs adsorbed with different concentrations (50, 250, 500 nM) of nanozymes after 1 hour incubation with Pro-Res.

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