"Metaphilic" Cell-Penetrating Polypeptide-Vancomycin Conjugate Efficiently Eradicates Intracellular Bacteria *via* a Dual Mechanism

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Materials and methods.

Materials: L-glutamic acid (99%), 6-chlorohexanol (96%), phosgene (15% in toluene), *O*-(2-Aminoethyl)-*O'*-(2-azidoethyl)pentaethylene glycol (N_3-PEG6-NH_2, > 90%), 2-propynylamine (98%), H-pyrazole-1-carboxamidine hydrochloride (99%), diisopropylethylamine (> 99%), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (> 97%), N-hydroxysuccinimide (98%), DBCO-Cy5, 5 (6)-carboxyfluorescein (> 95%) were ACS or HPLC grade reagents purchased from Sigma Aldrich, used as received. Silica gel (particle size 40-63 μm) was purchased from SiliCycle Inc. (Quebec City, Quebec, Canada) and desiccated by heating to 150 °C for 48 h before use. Lipids 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DOPG), 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (sodium salt) (18:1 cardiolipin), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids, Inc, used as received. γ-(6-chlorohexyl)-L-glutamate (CHLG) and the N-carboxyanhydride (NCA) of CHLG, CHLG-NCA, were synthesized according to our previously published work.[1-2] All other chemicals and solvents are ACS or HPLG grade reagents purchased from certified vendors such as Sigma Aldrich, TCI America or Fisher Scientific, and used as received unless otherwise specified. LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells, Lysotracker™ Green DND-26, CellMask™ Orange, Hoechst 33342 and SYTO™ 9 Green was purchased from Thermo Fisher Scientific Inc, used according to manual instruction. Bacteria strains, *Staphylococcus aureus* (ATCC 12608), methicillin-resistant *Staphylococcus aureus* (MRSA, USA 100) and vancomycin-resistant *enterococci* (VRE, ATCC 51858) and mammalian cell line RAW 264.7 macrophage, were purchased from ATCC. Strains/cells were reactivated and stored according to ATCC instructions. Human red blood cells (HRBCs) were purchased from Innovative Research, stored at 4°C and used within 30 days.

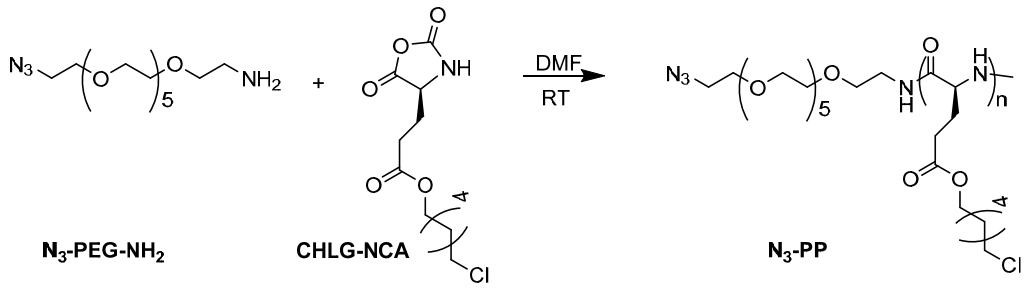
Animals: Female mice CD1-IGS (6-8 weeks old) were purchased from Charles River (Wilmington, MA, USA). Food and water were available ad libitum. Artificial light was provided in a 12 h/12 h cycle. The animal protocol was reviewed and approved by the Illinois Institutional Animal Care and Use Committee (IACUC) of University of Illinois at Urbana-Champaign.

Methods: ^{1}H NMR spectra were recorded on a Varian U500 (500 MHz) or UI400 (400 MHz) NMR spectrometer. The molecular weights of polypeptides were characterized by a gel permeation chromatography (GPC) system, which includes an isocratic pump (Model 1200 Infinity II, Agilent Technology), an autosampler (Waters 117 Plus Autosampler, Waters), a DAWN HELEOS multi-angle laser light scattering detector (MALLS) detector (Wyatt Technology), and an OptilabrEX refractive index detector (Wyatt Technology). Samples were separated by running through a serially connected size exclusion columns (100 Å, 10E3 Å, 10E4 Å, and 10E5 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex) at 50 °C with DMF containing 0.1 mol/L LiBr as the mobile phase. The Molecular weights and size distribution of each polymer were analyzed using ASTRA software (version 6.1, Wyatt Technology) based on the dn/dc value determined by the established methods come with the software. Circular dichroism (CD) spectra were measured by a JASCO J-815 CD spectrometer. Samples were diluted to predetermined concentrations (usually ~0.03 mg/mL) with Millipore water, and added into a quartz cell (1 cm × 1 cm) for measurement. A Perkin Elmer 100 serial FTIR spectrophotometer was used to measure the infrared spectra of samples. Samples were dissolved in a volatile solvent (acetone or chloroform) and then coated onto a KBr disk. HPLC analyses were performed on a Shimadzu CBM-20A system (Shimadzu, Kyoto, Japan) equipped with SPD20A PDA detector (190 nm-800 nm) and RF10Axl fluorescence detector, and an analytical C18 column (Shimadzu, 3 μm, 50 × 4.6 mm, Kyoto, Japan).

Experiments

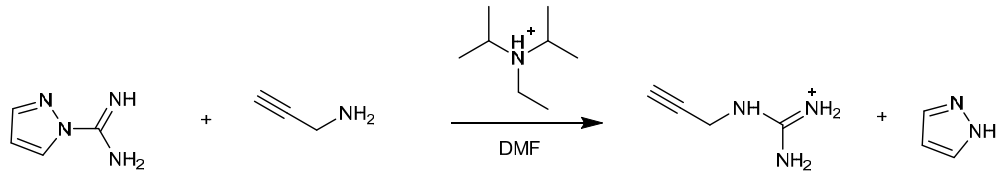
1. Synthesis

Synthesis of N_3-PEG-PCHLG (N_3-PP): Azido-functionalized PCHLG was synthesized with N_3-PEG-NH_2 (six ethylene glycol length) as the initiator in DMF (Scheme S1). Briefly CHLG-NCA (100 mg, 0.34 mmol), and N_3-PEG-NH_2 (11.9 mg, 0.034 mmol) were dissolved in DMF (1 mL) in a glovebox. The mixture was stirred under room temperature for 18 h before precipitating into hexanes/ether = 1/1 (v/v) twice. The product was collected by centrifugation and dried in vacuum. The structure of N_3-PP was confirmed by ^{1}H NMR (Figure S1a).



Scheme S1. Synthesis of N_3-PEG-PCHLG (N_3-PP).

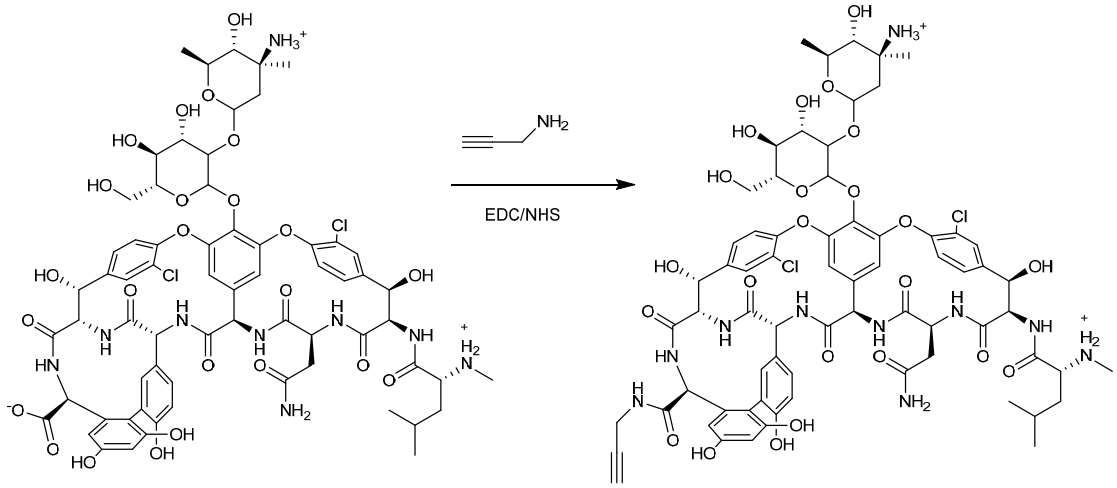
Synthesis of 2-propynylguanidinium. 2-propynylguanidinium was synthesized according to literature.[3] Briefly, 2-propynylamine (0.135 g, 2.5 mmol), H-pyrazole-1-carboxamidine hydrochloride (0.367 g, 2.5 mmol) and diisopropylethylamine (0.32 g, 2.5 mmol) were dissolved in anhydrous DMF (3 mL) (Scheme S2). The mixture was allowed to stir at room temperature for 24 h before precipitating in ether twice. The product was re-dissolved by water and lyophilized. The structure of 2-propynylguanidinium was confirmed by ^{1}H NMR. ^{1}H NMR (DMSO-d_6): δ 8.5-7.0 ppm (m, 5H, guanidinium protons), δ 4.02 ppm (s, 2H, -C***H***$_2$-), δ 3.34 ppm (s, 1H, -CC***H***).



Scheme S2. Synthesis of 2-propynylguanidinium.

Synthesis of propynyl-vancomycin: Propynyl-vancomycin was synthesized by EDC coupled amide formation (Scheme S3). Vancomycin (0.145 g, 0.1 mmol) was dissolved in DMF (5 mL) in a 20-mL vial. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (57.5 mg, 0.1 mmol), N-hydroxysuccinimide (34.5 mg, 0.3 mmol), triethylamine (91 mg, 0.9 mmol) and 2-propynylamine (55.4 mg, 0.93 mmol) dissolved in DMF (5 mL) were added into the vial in ice bath. The mixture was stirred at room temperature for 2 days before precipitating in ether twice. The product was purified by passing through a C18 reverse column with gradient mixture of acetonitrile/water (5-15% acetonitrile, with 0.1% TFA) as the eluant. The structure of propynyl-vancomycin was confirmed by ^{1}H NMR (Figure S2). In addition to NMR, the structure was

further confirmed by mass spectrum (Figure S3) and the purity was determined to be 96% by HPLC (Figure S4).



Scheme S3. Synthesis of propynyl-vancomycin.

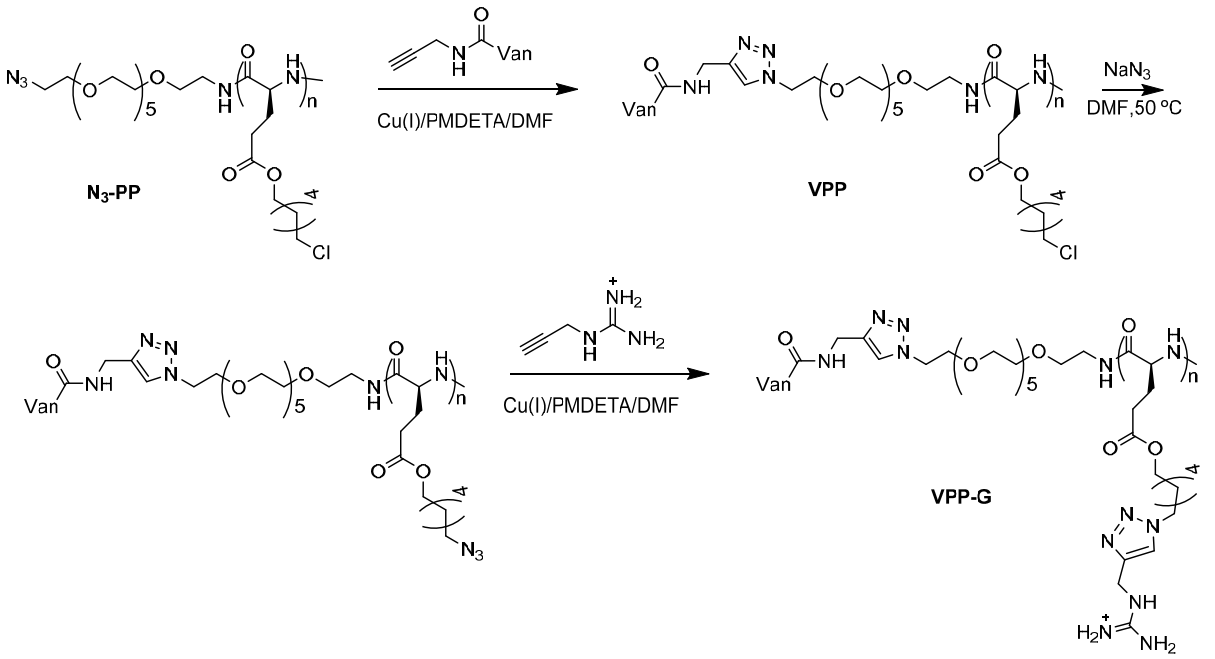
Synthesis of Van-PEG-PCHLG-Guanidinium (VPP-G): Vancomycin-functionalized N_3-PEG-PCHLG (VPP) was synthesized via copper-catalyzed click reaction in glovebox (Scheme S4). The molar ratio of N_3-PP/Van/PMDETA/CuBr is 1/1.5/1/1. For example, N_3-PEG-PCHLG (20 mg, 0.0072 mmol), propynyl-vancomycin (16 mg, 0.011 mmol), PMDETA (1.2 mg, 0.0072 mmol), and CuBr (1.0 mg, 0.0072 mmol) were dissolved in DMF (2 mL) in an oxygen-free glovebox. The mixture was stirred in room temperature for 24 h. Impurities and unconjugated vancomycin were then removed by dialysis in DI water. The white solid product was then collected by centrifugation and lyophilized before characterization by ^{1}H NMR (Figure S6).

Van-PEG-PAHLG was then synthesized by converting the side chain -Cl of Van-PEG-PCHLG to azido group (Scheme S4). For example, Van-PEG-PCHLG (20 mg, 0.047 mmol –Cl) and NaN_3 (30 mg, 0.47 mmol -N_3) were added into a vial with DMF (1 mL). The mixture was stirred at 50 ºC overnight. The remaining salt was removed by dialysis in DI water. The product Van-PEG-PAHLG was collected by centrifugation and lyophilized.

VPP-G was then synthesized by a second copper-catalyzed click reaction between Van-PEG-PAHLG and 2-propynylguanidinium (Scheme S4). The molar ratio of azido group/2-propynylguanidinium/CuBr/PMEDTA is 1/2/1/1. The click reaction was carried out in DMF in a glovebox. For example, Van-PEG-PAHLG (20 mg, 0.046 mmol -N_3), 2-propynylguanidinium

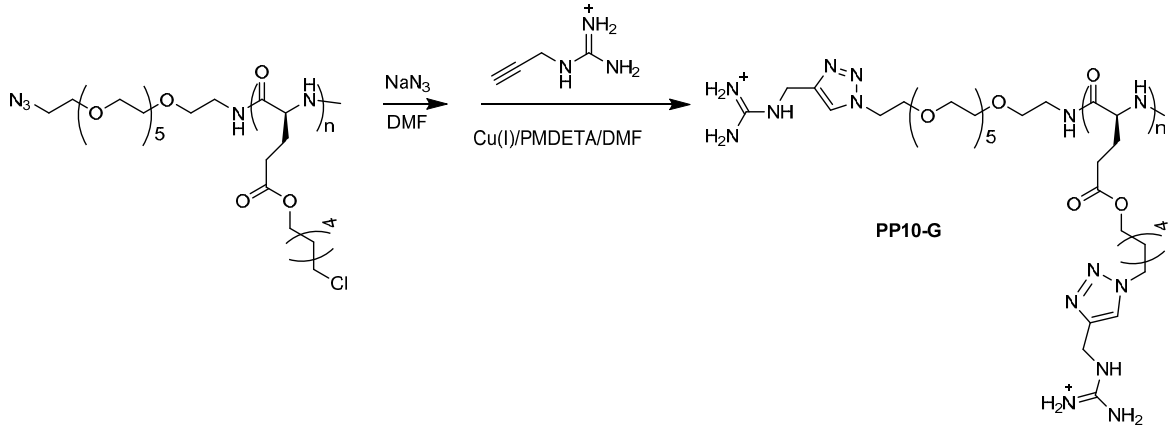
(9.8 mg, 0.1 mmol), PMDETA (8.0 mg, 0.046 mmol), and CuBr (6.6 mg, 0.046 mmol) were dissolved in 2 mL DMF in an oxygen-free glovebox. The mixture was stirred in room temperature for 24 h. Impurities were removed by dialysis in DI water. The final product was obtained after lyophilization and characterized by ^{1}H NMR (Figure S7).

To confirm that the antimicrobial activity of vancomycin was not affected by the reaction condition, free vancomycin (14.5 mg, 0.01mmol) was treated by NaN_3 (6.5 mg, 0.1mmol) in DMF (1 mL) at 50 ºC overnight. Salt was removed by dialysis and the product was recovered after lyophilization. Final product was subjected to antimicrobial activity evaluation (Figure S5).



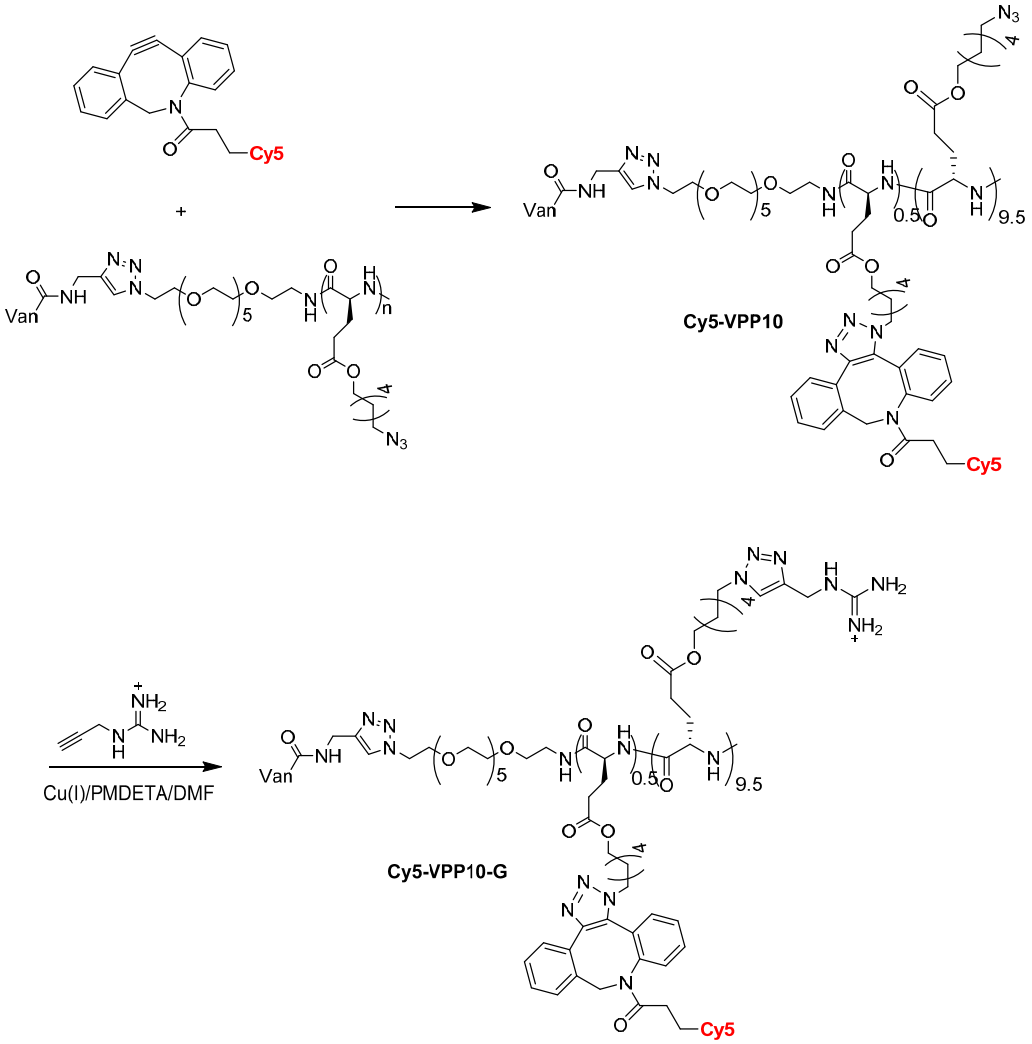
Scheme S4. Synthesis of Van-PEG-PCHLG-Guanidinium (VPP-G).

Synthesis of PEG-PCHLG-Guanidinium (PP-G): As a control, PEG-PAHLG-Guanidinium was also synthesized by similar method (Scheme S5). N_3-PEG-PCHLG was first converted to N_3-PEG-PAHLG by reacting with NaN_3 (10 eq) in DMF at 50 ºC for 24 h. Copper-catalyzed click reaction between N_3-PEG-PAHLG and 2-propynylguanidinium was then carried out at molar ratio of azido/2-propynylguanidinium/CuBr/PMEDTA = 1/2/1/1. The click reaction was carried out in DMF in glovebox. Impurities were removed by dialysis in DI water. The final product was obtained after lyophilization and characterized by ^{1}H NMR (Figure S8).



Scheme S5. Synthesis of PEG-PCHLG-Guanidinium (PP-G).

Synthesis of Cy5 labeled Van-PEG-PCHLG-Guanidinium (Cy5-VPP-G): Cy5 labeled Van-PEG-PCHLG-Guanidinium (Cy5-VPP-G) was synthesized by copper-free click reaction between DBCO-Cy5 and Van-PEG-PAHLG followed by copper-catalyzed click reaction between Cy5-VPP and 2-propynylguanidinium (Scheme S6). The molar ratio for copper-free click reaction is azido/DBCO-Cy5 = 20/1, while the molar ratio of copper-catalyzed click reaction is azido/2-propynylguanidinium/CuBr/PMEDTA = 1/2/1/1. Briefly, Van-PEG-PAHLG (16 mg, 0.38 mmol -N_3), DBCO-Cy5 (2.3 mg, 0.0019 mmol) were dissolved in DMF (2 mL) and stirred at room temperature overnight. The mixture was precipitated into ether and the product was collected by centrifuge. After removing solvent, the product was transferred into oxygen-free glovebox and re-dissolved by DMF solution (2 mL) containing 2-propynylguanidinium (7.4 mg, 0.75 mmol), PMDETA (6.5 mg, 0.038 mmol), and CuBr (5.4 mg, 0.038 mmol). The mixture was stirred in room temperature for 24 h. Impurities were then removed by dialysis in DI water. The final product was obtained after lyophilization.



Scheme S6. Synthesis of Cy5-VPP-G.

2. *In vitro* antimicrobial activity and toxicity

Antimicrobial activity against extracellular bacteria: Bacteria were inoculated in Luria-Bertani (LB) broth and cultured in a shaker incubator at 37 °C for 18 h. The suspension was then diluted into fresh LB broth (100 ×) for re-growth to mid-log stage (OD_{600} = 0.5-0.6). Bacterial suspension was diluted by fresh LB broth to ~5 × 10^5 CFU/mL, according to a pre-established optical density-CFU calibration curve. The stock solutions of Van, PP-G or VPP-G were made with sterile Millipore water. Additional serial 2-fold dilutions of the stock solutions with Millipore water gave a range of concentrations to be tested. To determine the MICs, diluted Van,

PP-G or VPP-G solutions (10 μL) and diluted bacterial suspension (90 μL) were added into corresponding wells in a preset 96-well plate. For the negative control, diluted bacterial suspension (90 μL) was mixed with sterile water (10 μL). For the positive control, sterile LB broth (90 μL) was mixed with sterile water (10 μL). The 96-well plates were then incubated at 37 °C for 18 h and the optical density at 600 nm (OD_{600}) was determined by a microplate reader (Perkin Elmer Victor^3 V). The bacterial growth ω was calculated by:

$$\omega = \frac{OD_{600,sample} - OD_{600,PC}}{OD_{600,NC} - OD_{600,PC}} \times 100\% ,$$

where $OD_{600,\ sample}$ is the optical density of each bacterial culture treated with a defined vancomycin or conjugate concentration. $OD_{600,\ NC}$ and $OD_{600,\ PC}$ are the optical density of the negative and positive control, respectively. The MIC is defined as the minimal concentration that completely inhibits bacterial growth (ω ≤ 5%), *i.e.*, no optical density difference was observed within experimental error when compared to the negative control. Each test was conducted at least triplicates and repeated on different days.

Antimicrobial activity against intracellular bacteria: RAW264.7 macrophages (3×10^5 cells) suspended in antibiotic-containing DMEM (supplemented with 10% 10× FBS and 1% 100× penicillin/streptomycin) were seeded in 24-well plate 24 h before the test. Bacterial cultures exhibiting logarithmic growth were collected by centrifugation (10,000 rpm for 5 min). The pelleted bacteria were re-suspended in antibiotic-free DMEM (supplemented with 10% 10× FBS only, no antibiotics) and incubated for 30 min at 37 °C for opsonization. Bacterial suspension was then adjusted to a concentration of 1.5×10^8 CFU/ml with antibiotic-free DMEM on the basis of a pre-established OD_{600}-CFU calibration curve. The supernatant of RAW 264.7 was removed and macrophages were washed with pre-warmed phosphate-buffered saline (PBS). Fresh antibiotic-free DMEM (500 μL) and bacterial suspension (5 μL, 1.5×10^8 CFU/ml) was added to each well. A multiplicity of infection (MOI, the number ratio of bacteria to macrophages) of 5 is used. The plate was incubated at 37 °C for 1 h to allow the phagocytosis of bacteria by macrophages. Macrophages were washed with PBS twice to remove extracellular bacteria and then exposed to gentamicin containing DMEM medium (100 μg/mL) for 2 h at 37 °C to further eradicate the extracellular bacteria. The cells were washed and replaced with antibiotic-free DMEM. Solutions of Van, PP-G and VPP-G at predetermined concentration were

then added to corresponding wells. The plate was incubated at 37 °C with 5% CO_2 for 24 h. Macrophages were then washed three times by PBS buffer and lysed with Triton X-100 (0.03%, 500 μL) by vigorous pipetting. Serial 10-fold dilution was immediately made in 15 min and the dilutions were spread on LB agar plate for overnight incubation. The CFU numbers yield from each tested concentration was compared to that of negative control (treated with PBS) for bacterial killing efficiency calculation. Each test was conducted at least triplicates and repeated on different days.

Hemolysis assay: The toxicity of Van, PP-G and VPP-G was tested by their hemolytic activities against HRBCs following established protocols.[6] Serial 2-fold dilutions of Van, PP-G and VPP-G with PBS buffer give a range of predetermined concentrations to be tested. Fresh HRBC suspension (1 mL) was washed twice with PBS buffer (12 mL) before re-dispersed in PBS buffer (15 mL) to reach a HRBC concentration of ~1.0% (v/v). Aliquots of this diluted HRBC suspension (160 μL) were mixed with diluted Van, PP-G and VPP-G solutions (40 μL) in 1.5 ml micro-centrifugation tubes. The tubes were secured in an shaker incubator and sharked at 250 rpm at 37 °C for 60 min. PBS buffer (40 μL) or Triton X-100 (40 μL, 1% v/v) mixed with HRBC suspension (160 μL) were as used as negative and positive controls, respectively. Cells and fragments were removed by centrifugation (1000× g, 5 min) and supernatant (30 μL) of each test sample was added into a 96-well plate with each well contains PBS buffer (100 μL). The absorbance at 405 nm was measured with a microplate reader (Perkin Elmer Victor^3 V). The percentage of hemolysis (ψ) was calculated by:

$$\psi = \frac{OD_{405,sample} - OD_{405,NC}}{OD_{405,PC} - OD_{405,NC}} \times 100\%,$$

Where $OD_{405,\ sample}$ is the absorbance of HRBC supernatant treated with a defined Van, PP-G or VPP-G concentration. $OD_{405,\ PC}$ and $OD_{405,\ NC}$ are the absorbance of positive and negative control, respectively. HC_{50} is defined as the minimal sample concentration that causes 50% hemolysis, which is commonly used as the index of toxicity.[6-8]

MTT cell viability assay: The toxicity of Van, PP-G and VPP-G was further assessed by MTT cell viability assay according to our previous work and literature.[9] RAW 264.7 macrophages (1×10^5 cells/mL, 100 μL) in sterilized DMEM (supplemented with 10% 10× FBS and 1% 100×

penicillin/streptomycin) was seeded into a preset 96-well plate and incubated at 37 °C with 5% CO_2 to reach 70% confluence. Van, PP-G and VPP-G solutions of predetermine concentration were prepared by serial 2-fold dilution of stock solution with sterile PBS buffer. The diluted drug solutions (10 μL) was added into the cell suspension and the plate was further incubated at 37 °C with 5% CO_2 for 24 h. Cells treated with PBS buffer (10 μL) or Triton X-100 solution (3%, 10 μL) were used as positive and negative control, respectively. After the incubation, MTT solution (10 μL, 5 mg/mL in sterile PBS buffer) was then added to each well and the plate was further incubated for another 4 h. The supernatant was removed and 100 μL DMSO was added to dissolve the formed purple crystal. The absorbance at 570 nm was measured on a plate reader (Perkin Elmer Victor^3 V). The percentage of cell death can be determined according to:

$$\gamma = 100\% - \frac{OD_{570,sample} - OD_{570,NC}}{OD_{570,PC} - OD_{570,NC}} \times 100\%$$

Where, $OD_{570,sample}$ is the absorbance of formazan at the presence of a defined drug concentration. $OD_{570,PC}$ and $OD_{570,NC}$ are the absorbance of the positive and negative control, respectively. IC_{50} is the minimal drug concentration that causes 50% of cell death.

Live/dead staining for mammalian cells: Live/dead staining assay was also employed to evaluate the cell toxicity of VPP-G at its effective concentration ($IMBC_{99.9}$). The assays were performed according to the vendor's instruction and literature.[10] The live/dead staining kit contains two fluorescent dyes: Calcein AM (ex/em: ~495/~515 nm) and ethidium homodimer-1 (ex/em: ~ 528/~617 nm). Calcein AM exhibits a green fluorescence only when it is degraded by live cells, while ethidium homodimer-1 enters cells only when their membranes is damaged and exhibits a red fluorescence after binding to nucleic acids. Consequently, live cells are stained with green, while dead cells are stained with red. RAW264.7 macrophages in antibiotics-containing DMEM (2×10^5 cells/mL, 2 mL) were seeded into a preset 6-well plate equipped with a sterile cover slide in each well. The plate was incubated at 37 °C with 5% CO_2 for 24 h. VPP-G stock solution was added into the cell suspension, so that the final concentration is 9 μM. Equal amount of Van (9 μM) or PP-G ((9 μM) were used as controls. The plate was further incubated for 24 h. For positive and negative control, equivalent volume of PBS buffer or Triton X-100 (final concentration 0.1% v/v) was added, respectively. After the incubation, the supernatant was removed and the cells were gently washed with PBS twice. DMEM (2 mL) with

Calcein AM (2 μM) and EthD-I (4 μM) was added to each well and incubated for another 30 min. The supernatant was then removed and cells were gently washed with PBS twice. The cover slides were placed on glass slides with Antifade Gold and imaged by confocal (Zeiss LSM 700 confocal microscope).

3. Mechanism study

Confocal imaging of cell penetration of Cy5-VPP-G: RAW264.7 macrophages (2×10^5 cells/mL, 2 mL) were seeded in a preset 6-well plate equipped with a sterile cover slide in each well. The plate was incubated at 37 °C with 5% CO_2 for 24 h. Cy5-VPP-G (ex/em: 649/666 nm) was diluted with sterile PBS buffer and added into each well so that the final concentration is 36, 18, 9, 4.5 and 0 μM. After incubating for 24 h, supernatant was removed and cells were washed with PBS twice to remove free Cy5-VPP-G. The supernatant was then replaced with fresh optimal-EM (2 mL). CellMask staining solution (2 μL, 1 mg/mL, ex/em: 554/567 nm) and Hoechst solution (2 μL, 10 mg/mL, ex/em: 350/461 nm) were added to the each well and incubate for 15 min. The supernatant was then removed and washed by PBS twice. Cells were then fixed with 4% PFA solution in PBS for 15 min. After washing by PBS twice, the cover slides were transferred to glass slides with Antifade Gold reagent and sealed with nail oil. Finally, the slides were imaged by confocal microscope (Zeiss LSM 700 confocal microscope).

Lysotracker colocalization: RAW264.7 macrophages (2×10^5 cells/mL, 2 mL) were seeded in a preset 6-well plate equipped with a sterile cover slide in each well 24 h before the assay. Cy5-VPP-G was diluted with sterile PBS buffer and added into each well so that the final concentration is 9 and 0 μM. After incubation for 24 h, supernatant was removed and cells were washed with PBS three times to remove free Cy5-VPP-G. The supernatant was then replaced with fresh optimal-EM (2 mL). Lysotracker green solution (2 μL, 50 μM, ex/em: ~504/511 nm) and Hoechst solution (2 μL, 10 mg/mL) were added to the each well and incubate for 30 min. The supernatant was then removed and the cells were washed by PBS twice. The cells on cover slides were fixed with 4% PFA solution in PBS for 15 min. After washing by PBS twice, the cover slides were transferred to glass slides with Antifade Gold reagent and sealed with nail oil. Finally, the slides were then imaged by confocal microscope (Zeiss LSM 700 confocal microscope). The rest of cells in the 6-well plate were recovered by trypsin-EDTA (0.25%

trypsin/0.53 mM EDTA) digestion for 5 min before fixation with 4% PFA solution for overnight and followed by flow cytometry analysis.

SYTO9 stained bacteria colocalization: RAW264.7 macrophages (2×10^5 cells/mL, 2 mL) were seeded into a preset 6-well plate equipped with a sterile cover slide in each well 24 h before the assay. The medium was replaced with antibiotic-free DMEM (supplemented with 10% of 10× FBS solution, no antibiotics) 30 min before the assay. Bacterial cultures in logarithmic growth were collected by centrifugation at 10,000 rpm for 5 min, washed and resuspended in PBS. The bacterial suspension was adjusted to 6×10^8 CFU/mL ($OD_{600} = 1.2$) and stained with SYTO9 (5 μL, 5 mM, ex/em: 483/503 nm) for 15 min. The bacteria were washed by PBS three times and collected by centrifugation (10,000 rpm, 5 min). RAW264.7 cells were infected with SYTO9 labelled MRSA at a MOI of 5 for 1 h. The extracellular bacteria were washed away by PBS for three times. Cy5-VPP-G was diluted with sterile PBS buffer and added into each well so that the final concentration is 9 and 0 μM. After incubation for 24 h, supernatant was removed and cells were washed with PBS for three times to remove free Cy5-VPP-G. The supernatant was then replaced with fresh optimal-EM growth media (2 mL). Hoechst solution (2 μL, 10 mg/mL) was added to the each well and incubate for 15 min. Supernatant was removed and cells were washed by PBS twice. The cells on cover slides were fixed with 4% PFA solution in PBS for 15 min. After washing by PBS twice, the cover slides were transfered to glass slides with Antifade Gold reagent and sealed with nail oil. Finally, the slides were then imaged by confocal microscope (Zeiss LSM 700 confocal microscope). The rest of cells in the 6 well plate were recovered by trypsin-EDTA (0.25% trypsin/0.53 mM EDTA) digestion for 5 min before fixation with 4% PFA solution in PBS overnight and followed by flow cytometry analysis.

Liposome dye leakage: Small unilamellar vesicles (SUVs) with lipid compositions mimicking bacterial membranes were prepared as previously reported.[11-12] Briefly, DOPG and cardiolipin (CL) were dissolved in chloroform and mixed at a molar ratio of DOPG/CL = 50/50 to mimic *S. aureus* lipid bilayer. The organic solvent was evaporated by a N_2 stream, and the resultant lipid film was further dessicated in vacuum. Solution of 5 (6)-carboxyfluorescein (40 mM), a concentration dependent self-quenching dye, was added to hydrate the lipid films and the final liposome concentration was adjusted to be 10 mg/mL. After incubation in 37 °C overnight, the

liposome solution was sonicated by a probe sonicator to clarity and extruded through an Avanti mini-extruder set equipped with a polycarbonate membrane (0.1 μm pore size) for 11 times to obtain uniform SUVs. The free dye was removed by passing through PD10 column and elute with 80 mM NaCl according to vendor instruction. Similarly, mammalian membrane mimicking liposomes were prepared by mixing DOPC and cholesterol at a molar ratio of DOPC/cholesterol = 60/40. Dye leakage was then performed in dark with a fluorometer. Liposome solution (20 μL) was diluted with NaCl solution (80 mM, 3 mL). The fluorescent intensity before and after the addition of Van, PP-G or VPP-G solutions (9 μM) was recorded as a function of time. Completed dye leakage (100%) was reached after addition of Triton X-100 (0.1% v/v).

Resistance tests: The resistance tests were performed similar to bacteria inhibitory tests. In summary, mid-log stage (OD_{600} = 0.5-0.6) bacteria was diluted to ~5×10^5 CFU/mL (OD_{600} = 0.001) and mixed with prediluted solutions of vancomycin and VPP-G. The MICs were determined after overnight incubation. The bacteria survived from sub-inhibitory concentration of vancomycin and VPP-G were used for subsequent MIC determination following the same method. This process was repeated for at least 20 passages.

Characterization of cell wall inhibition by SEM: *S. aureus* was inoculated into LB broth and grown to mid-log phase (OD_{600} = 0.5-0.8). Bacterial suspensions were diluted to OD_{600} = 0.05 (2.5×10^7 CFU/mL) with sterile LB and mixed with pre-diluted vancomycin or VPP-G solutions so that the final concentration ratio of drug/bacteria equivalent to the ratio used for MIC determination. Bacteria mixed with PBS were used as negative control and bacteria treated with PP-G were used to visualize the effects on CPPs by itself. The mixture was allowed to incubate for 12 h in a shaker incubator. Bacteria cells were then collected by filter membrane (100 nm pore size, Whatman) and fixed with 4% PFA for 4 h. Finally, the bacterial cells were dehydrated with 50%, 75%, 90%, and 100% ethanol solutions, each for 15 min. Solvent were removed by lyophilization. The dessicated membranes were cut into small pieces, placed onto a carbon tape fixed on a steel stub and coated with 10 nm gold before imaging. Images were taken on a Hitachi 4800 high resolution SEM at accelerate voltage of 10 kV and probe current of 5 μA.

4. *In vivo* antimicrobial activity and toxicity

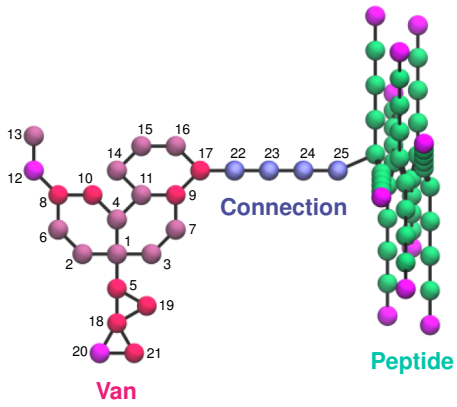
***In vivo* antimicrobial activity**: Mice (CD1-IGS) were randomly divided into 4 groups ($n \geq 6$ for each group): PBS treated control group, vancomycin treated control group, PP-G treated control group and the VPP-G treated control group. All mice were infected with 200 μL MRSA suspension (USA100, 1×10^8 CFU/mL) through tail vein intravenous injection. Either PBS (100 μL), vancomycin solution (100 μL, 100 mg/kg), PP-G (100 μL, 50 mg/kg) or VPP-G solution (100 μL, 75 mg/kg) was administrated through intraperitoneal injection 24 h after infection. Second dosage was administrated 12 h after the first dosage, same amount. All mice were sacrificed 36 h after the last injection (72 h after the infection) and their kidneys, liver, heart and spleen were collected. Organs were divided into two parts. One-fourth of the organ were fixed by 20% neutral buffered formalin for histology analysis, the other rest tissues were then suspended in 5 mL PBS buffer with 0.1% Triton X-100 for homogenization. The lysate was then serial 10-fold diluted with PBS buffer and aliquots (10 μL) were spread on LB agar plates for CFU determination. Mouse body weight was monitored daily right after the infection.

Histology analysis: The *in vivo* tissue toxicity and therapeutic efficacy were evaluated by H&E staining. In addition to the tissues collected from infected mice as described above, tissues from uninfected mice were used as a control. Tissues were fixed with 20% neutral buffered formalin for 24 h after collecting from the mice, followed by dehydration in 70% ethanol before embedding with paraffin. The embedded tissues were then cut into sections of 4 μm with a Leica RM2125 RTS microtome. Sections were stained with Hematoxylin solution (Thermo Scientific™ Richard-Allan Scientific™ Modified Harris Hematoxylin) and Eosin/Phloxine solution (Thermo Scientific™ Richard-Allan Scientific™ Eosin-Y Alcoholic, Eosin-Y with Phloxine, Eosin-Y Saturated) according to the user manual. The stained slides were then analyzed by a certified expert.

5. Simulation

We performed coarse-grained molecular dynamics (MD) simulations using the LAMMPS package to investigate the landing and subsequent insertion process of a VPP-G on and into a cell lipid membrane. We employed a similar coarse-graining scheme as developed previously.[13] All molecules are represented as assemblies of spherical beads of diameter $\sigma = 8.5$ Å. In

particular, the lipid molecule is modeled as a 4-bead-long rigid rod. The membrane contains 20% of the lipids carrying a negative charge –1e on their headgroup. The VPP-G molecule is composed of guanidinium-functionalized helical polypeptide and a vancomycin (Van) molecule connected by an oligo(ethylene-glycol). The coarse-grained representation is shown in Scheme S7. The 10-unit long polypeptide is modeled as a rigid helix grafted with 10 flexible side chains, each of which carries a positive charge +1e on the endgroup. The Van molecule contains 21 beads, connected to the peptide sequence via a 4-bead-long connection linker.



Scheme S7. The coarse-grained model of the VPP-G molecule. The VPP-G molecule is a short helical polypeptide (green) concatenated by a vancomycin molecule (red) with an oligo(ethylene-glycol) linker (blue). In aqueous environment, the VPP-G molecule is positively charged. All the cationic groups (pink) carry a positive charge +1e. Both the helical core of the polypeptide (10 beads) and the core of the Van molecule (beads 1-5) were treated as a rigid body. According to the chemical formulas, we assigned the dark red beads 1-4, 6-7, 11-16 to the Van molecule and all the green beads to the hydrophobic groups of the helical polypeptide.

We maintain the sizes of the molecules by applying a strong harmonic bond potential to all connections between the beads,

$$U_{\text{bond}}(r) = -k_{\text{bond}}\,(r - r_0)^2\,,$$

with equilibrium bond length $r_0 = 1.2\sigma$ and strength $k_{\text{bond}} = 300 k_{\text{B}}T/\sigma^2$.

We also take into account the rigidity of the molecules. The helical core of the polypeptide as well as the planar core of the Van molecule (beads 1–5 in the model schematics S7) are treated as a rigid body. A strong harmonic angle potential is introduced to represent the local rigidity of the molecules,

$$U_{\text{angle}}\,(\theta) = -k_{\text{angle}}\,(\theta - \theta_0)^2\,,$$

with k_{angle} =10k_BT /rad^2. For the Van molecule, the angle potential is applied among the following angles defined by 3 adjacent beads:

Bead-bead-bead	Equilibrium angle (θ_0)
1-2-6	135°
1-3-7	135°
1-4-10	135°
1-4-11	135°
1-5-18	180°
5-18-20	120°
5-18-21	120°
6-8-12	135°
8-12-13	135°
10-8-12	135°
11-14-15	90°
14-15-16	135°
15-16-17	135°

For lipids, the angle potential is applied between all two adjacent bonds with an equilibrium angle $\theta_0 = 180°$.

In addition to the bonded interactions listed above, all beads experience excluded-volume and Coulombic interactions. The former are implemented via a shifted-truncated Lennard-Jones (LJ) potential with strength $\varepsilon = 0.7k_BT$ and cut-off $r_c = 2^{1/6}\sigma$, while the latter is treated via Ewald summation with a relative accuracy of 10^{-4}.

To account for the hydrophobicity of the molecules, we employ a generic solvent-free model. All hydrophobic components (the green beads on the peptide, the dark red beads on the Van molecule, and the tail of the lipid) have an effective attraction,

$$U(r) = -\varepsilon \cos^2[\pi(r - r_c)/2w_c], \quad r_c \le r \le r_c + w_c ,$$

with $w_c = 1.6\sigma$. We choose a small attraction strength $\varepsilon = 0.7k_BT$ so that the lipid molecules form a stable but still liquid-like cell membrane.

To quantify the driving force of the landing process, we performed a steered MD simulation. We systematically varied the distance between the VPP-G molecule and the membrane. At any given separation z, the center of mass of the VPP-G molecule was fixed yet all other degrees of

freedom were free to change. This allows us to calculate the potential of mean force as a function of z and thereby derive the free-energy change involved in the landing process. We also simulated the subsequent insertion process of the VPP-G molecule into the cell membrane. To allow reconfiguration of the membrane during insertion, we applied a Berendsen barostat and kept the system under constant pressure, set equal to the osmotic pressure of a 100 mM salt solution. The dimensions of the system were $60\times60\times60\sigma^3$, containing 7,200 lipids and over 17,000 ions. Periodic boundary conditions were applied in all three dimensions. All simulations were performed for more than 10^7 time steps, with time step $dt = 0.002\tau$, where $\tau = (m\sigma^2/\varepsilon)^{1/2}$ (m the bead mass) is the LJ time unit.

6. Statistical analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) with post hoc Fisher's LSD test (Origin Pro 2018b), or student Student's t-test (two-tailed) comparisons at 95% confidence interval. P-values < 0.05 were considered statistically significant. The results were deemed significant at $0.01 < *P \leq 0.05$, highly significant at $0.005 < **P \leq 0.01$, and extremely significant at $***P \leq 0.005$.

7. Safety statement

No unexpected or unusually high safety hazards were encountered.

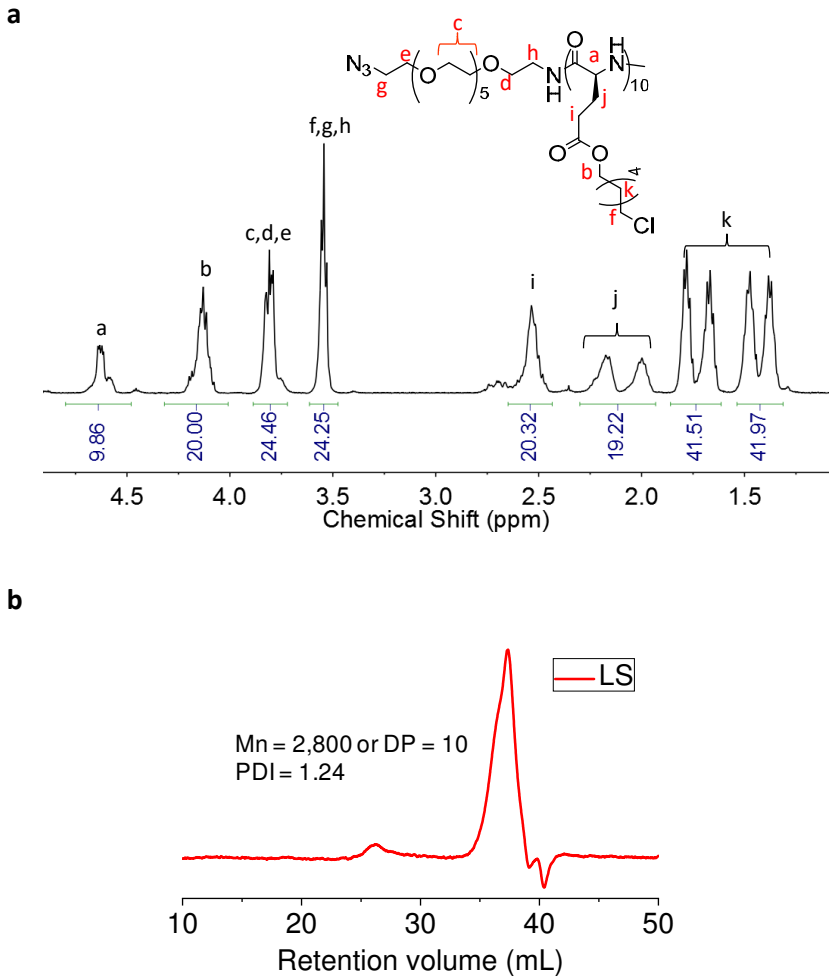


Figure S1. Characterization of N_3-PP. (a) ^{1}H NMR of N_3-PP in $CDCl_3$/TFA-d_4 = 85/15. Using the initiator peak (**c,d,e**) as a reference, the degree of polymerization (DP) is determined to be ~10. (b) GPC trace of N_3-PP. DMF with 0.1 M LiBr was used as the mobile phase. The molecular weight *Mn* and PDI are determined to be 2,800 Da (DP of 10) and 1.24, respectively.

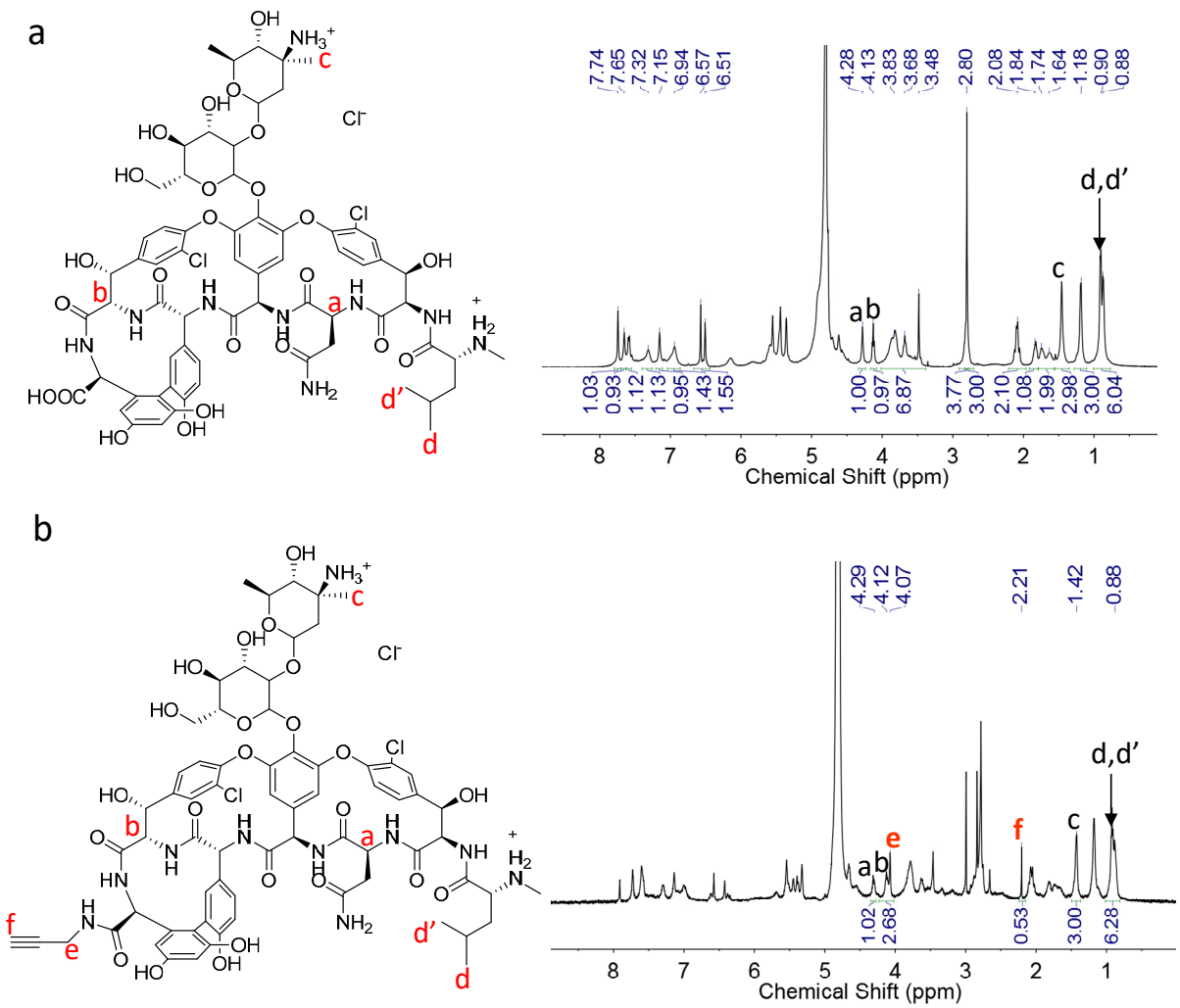


Figure S2. Characterization of propynyl-vancomycin. a) ^{1}H NMR of vancomycin in deuterium oxide. b) ^{1}H NMR of propynyl-vancomycin in deuterium oxide. All peaks can be assigned according to literature,[4-5] only characteristic peaks are shown for simplicity. The protons from propynyl moiety are identified as peak **e** and **f**.

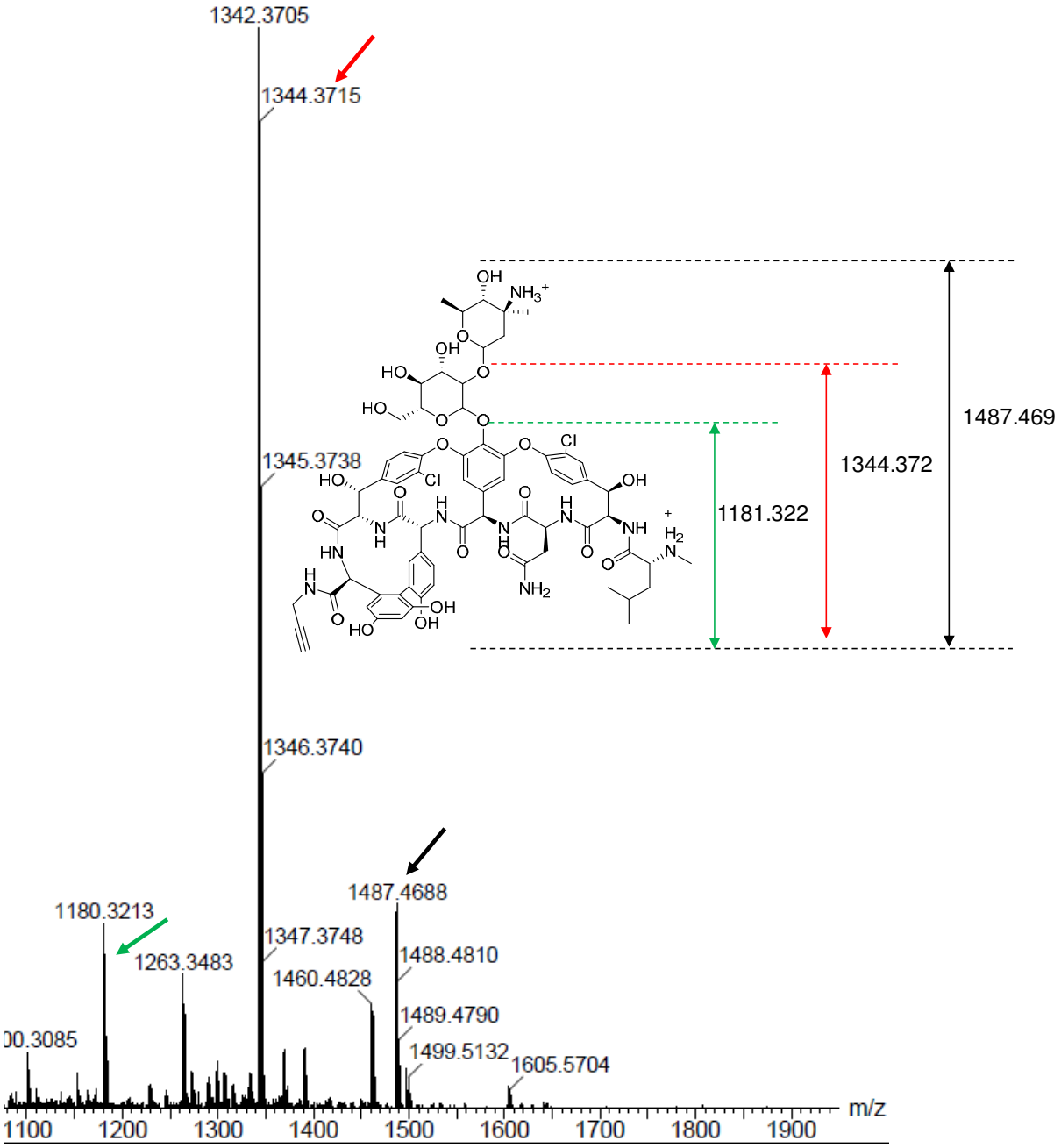


Figure S3. Mass spectrum of propynyl-vancomycin. The peaks identified from the mass spectrum matched with the calculated molecular weight of three major fragments: whole molecule (1487.469 Da), fragments without L-vancosamine (1344.372 Da), and fragments without L-vancosamine and D-glucose (1181.322 Da).

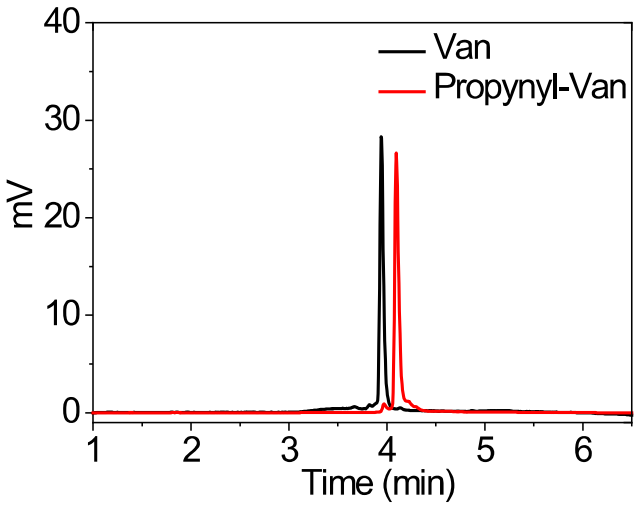


Figure S4. HPLC trace of vancomycin (Van) and propynyl-vancomycin (propynyl-Van). The purity of propynyl-vancomycin was determined to 96%.

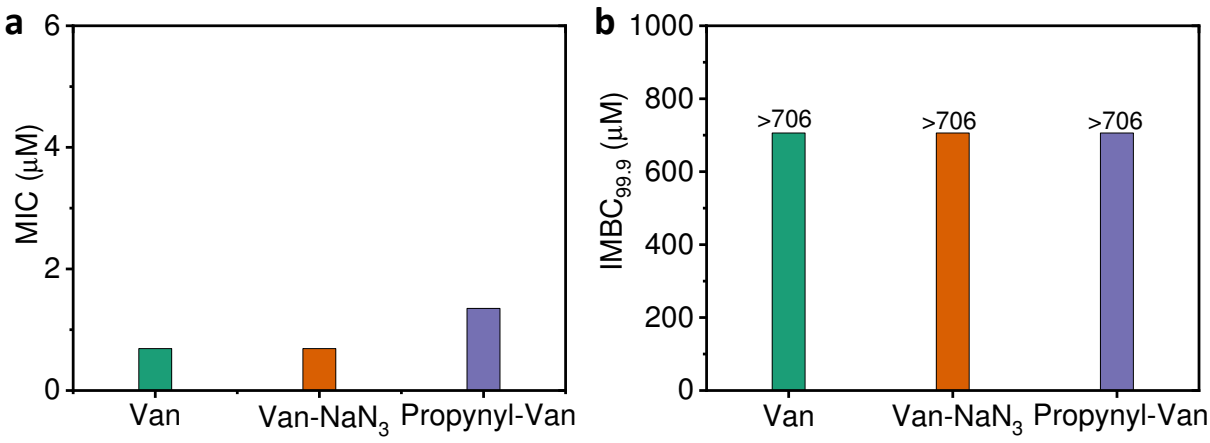


Figure S5. MIC (a) and $IMBC_{99.9}$ (b) of vancomycin (Van), vancomycin treated by NaN_3 at 50 ºC for 24 h (Van-NaN_3), and propynyl-vancomycin (propynyl-Van) against extracellular and intracellular *S. aureus* (ATCC12608), respectively

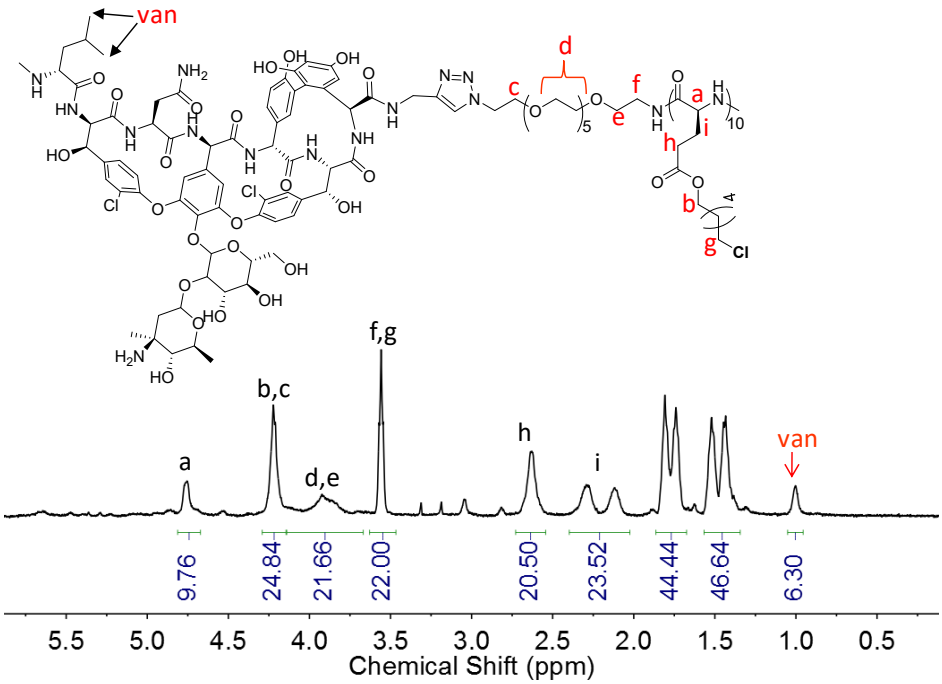


Figure S6. ^{1}H NMR of VPP in $CDCl_3$/TFA-d_4 = 85/15. The characteristic peak of vancomycin is indicated in red arrow. When peak (**f,g**) is set to be 22 protons, the characteristic peak of vancomycin has 6 protons, indicating that each polypeptide chain is grafted with one vancomycin molecule.

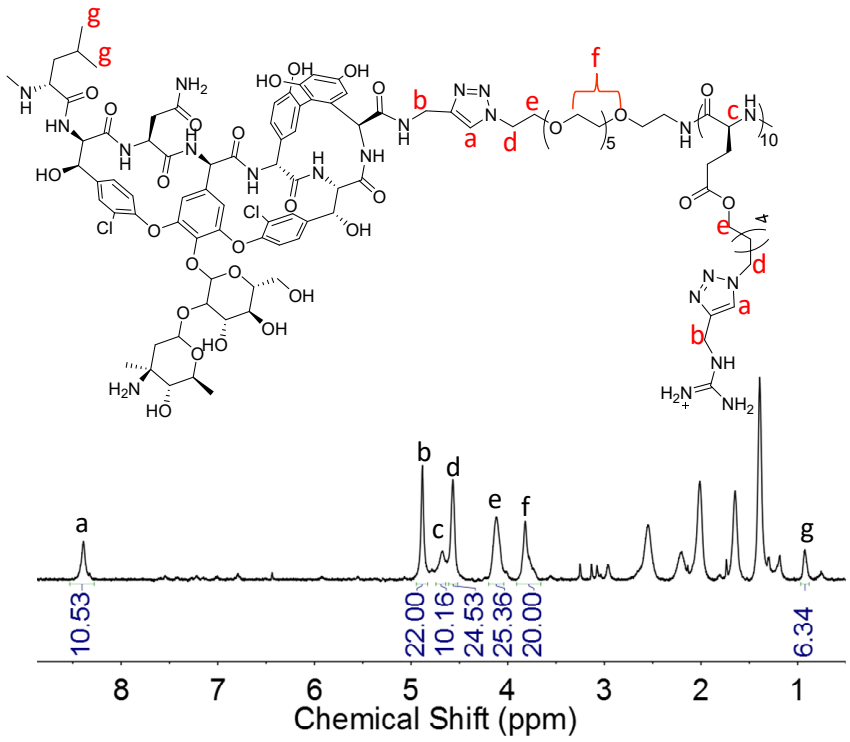


Figure S7. ^{1}H NMR of VPP-G in TFA-d_4. When peak **f** is set to be 20 protons, the characteristic peak of vancomycin peak **g** has 6 protons and the characteristic peak of triazole moieties peak **a** has 11 protons, suggesting that each polypeptide chain has been grafted with one vancomycin molecule and all the side chains were grafted with guanidinium.

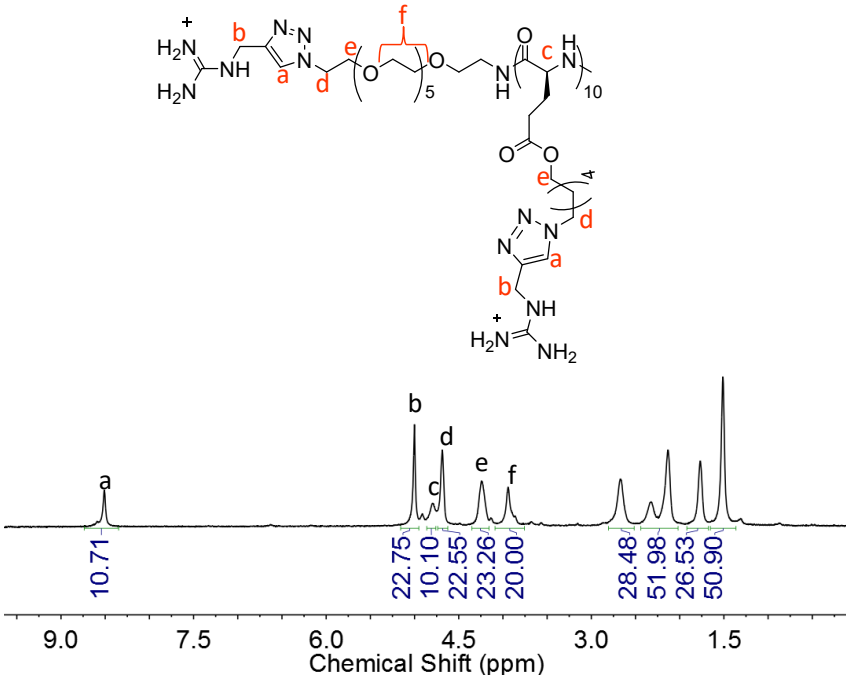


Figure S8. ^{1}H NMR of PP-G in TFA-d_4. When peak **f** is set to be 20 protons, peak **b** has 22 protons and the characteristic peak of triazole moieties peak **a** has 11 protons, suggesting that each polypeptide chain was grafted 11 guanidinium moieties.

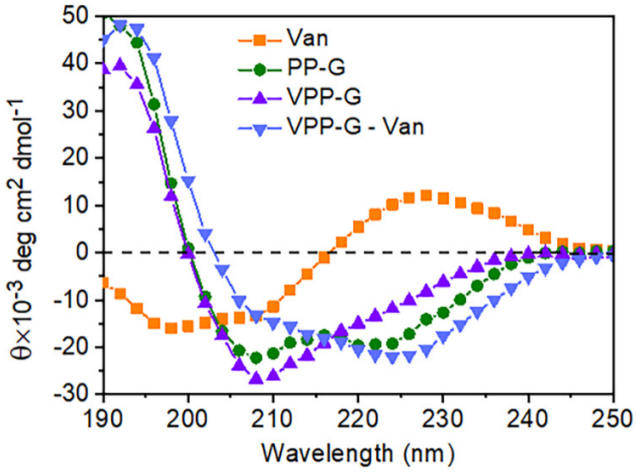


Figure S9. CD spectra of vancomycin (orange), PP-G (olive) and VPP-G (purple). The cyan curve is the differential spectrum obtained by substracting vancomycin spectrum from VPP-G spectrum.

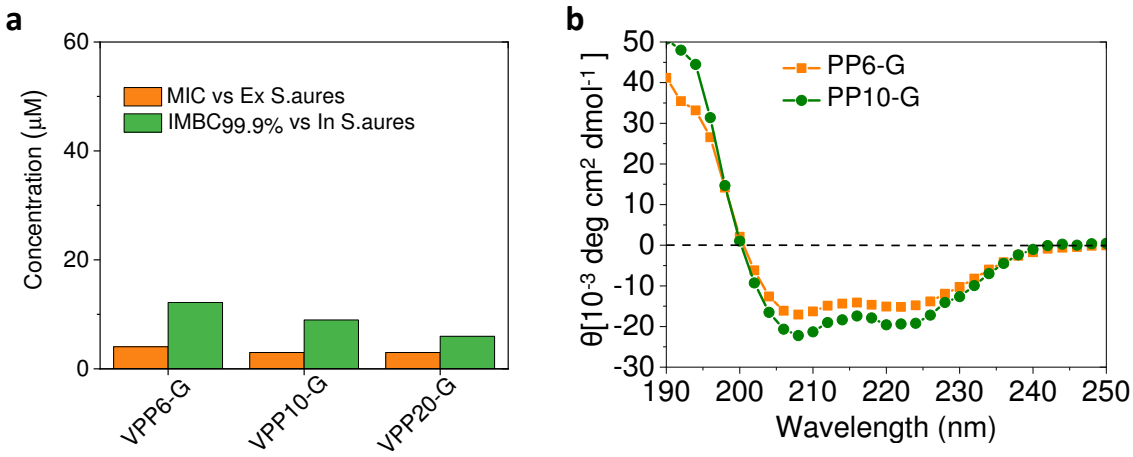


Figure S10. (a) The extracellular and intracellular antimicrobial activity of VPP6-G (DP of 6), VPP10-G (DP of 10), and VPP20-G (DP of 20) against *S. aureus* (ATCC12608). (b) The CD spectra of PP6-G and PP10-G in water.

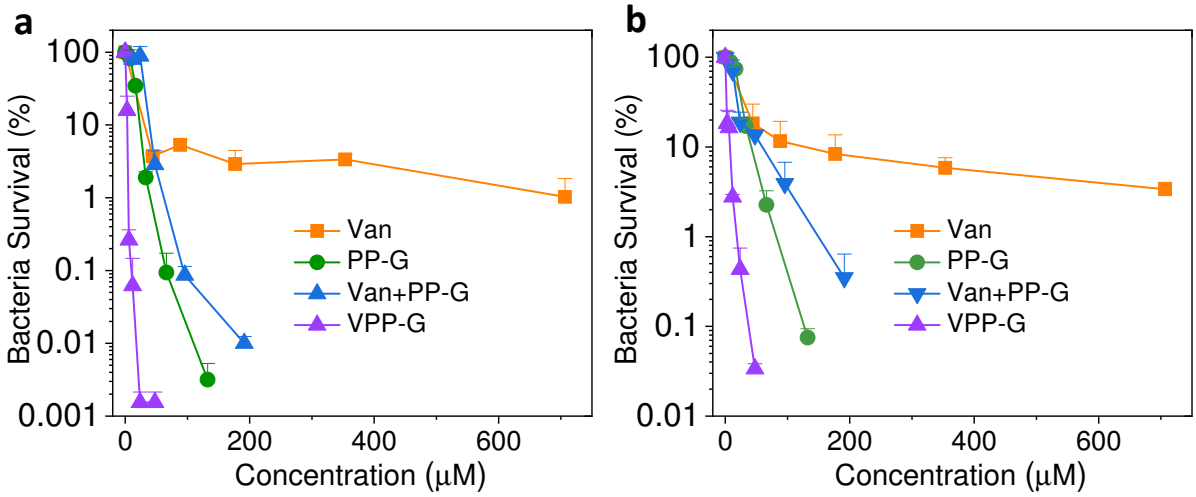


Figure S11. Intracellular survival rate of (a) MRSA (USA100) and (b) VRE (ATCC51858) treated with vancomycin (Van), PP-G, Van + PP-G or VPP-G.

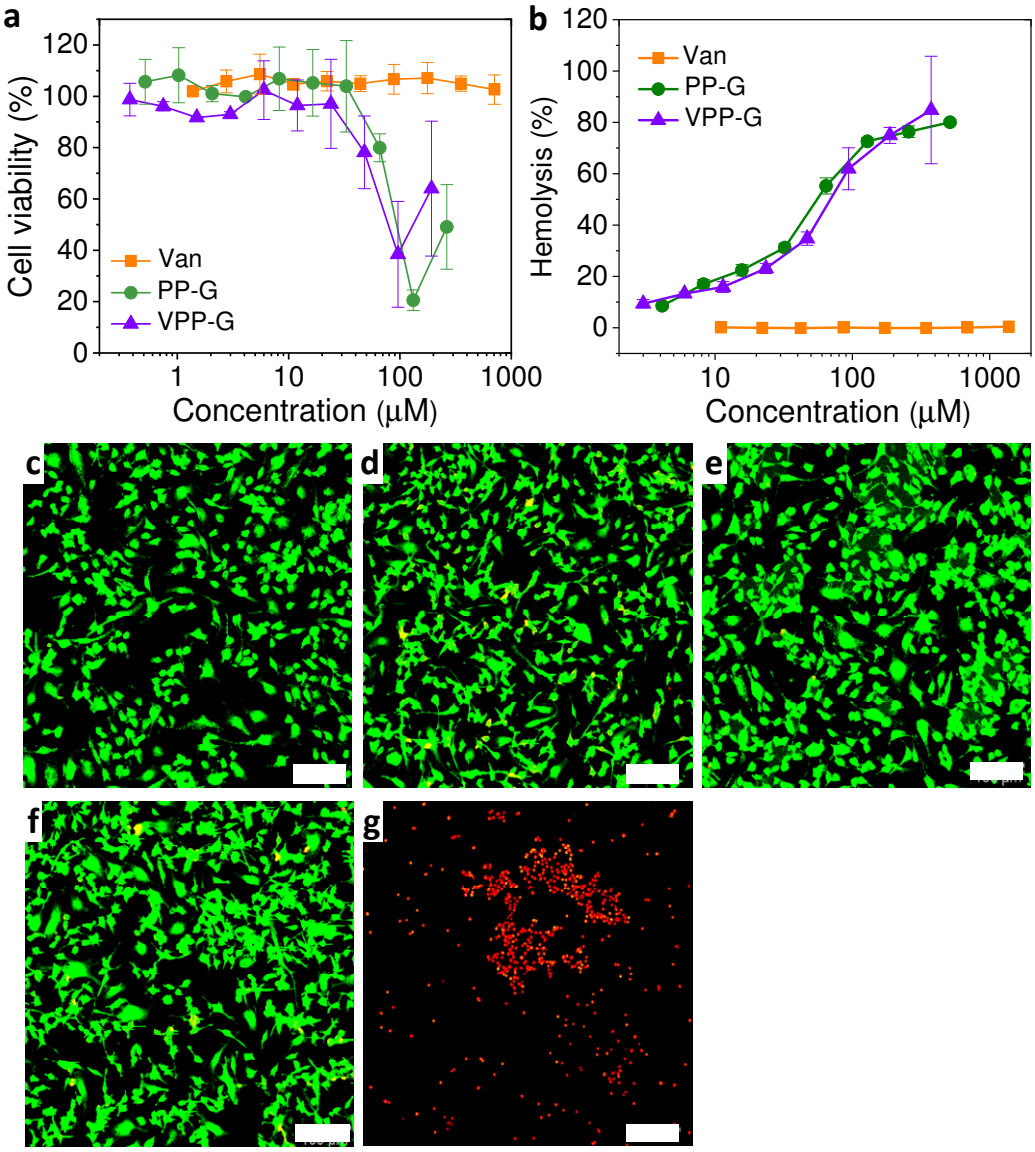


Figure S12. In vitro cytotoxicity of VPP-G and its controls. (a) Cell viability of RAW264.7 macrophages treated with VPP-G and controls. IC_{50} is defined as the lowest concentration causing 50% of cell death. (b) Hemolysis of human red blood cells treated with VPP-G and controls. HC_{50} is defined as the lowest concentration causing 50% of hemolysis. Live/dead staining of macrophage RAW 264.7 treated with (c) PBS, (d) vancomycin (9 μM), (e) PP-G (9 μM), (f) VPP-G (9 μM), and (g) Triton X-100 (0.1%). Scale bar: 100 μm.

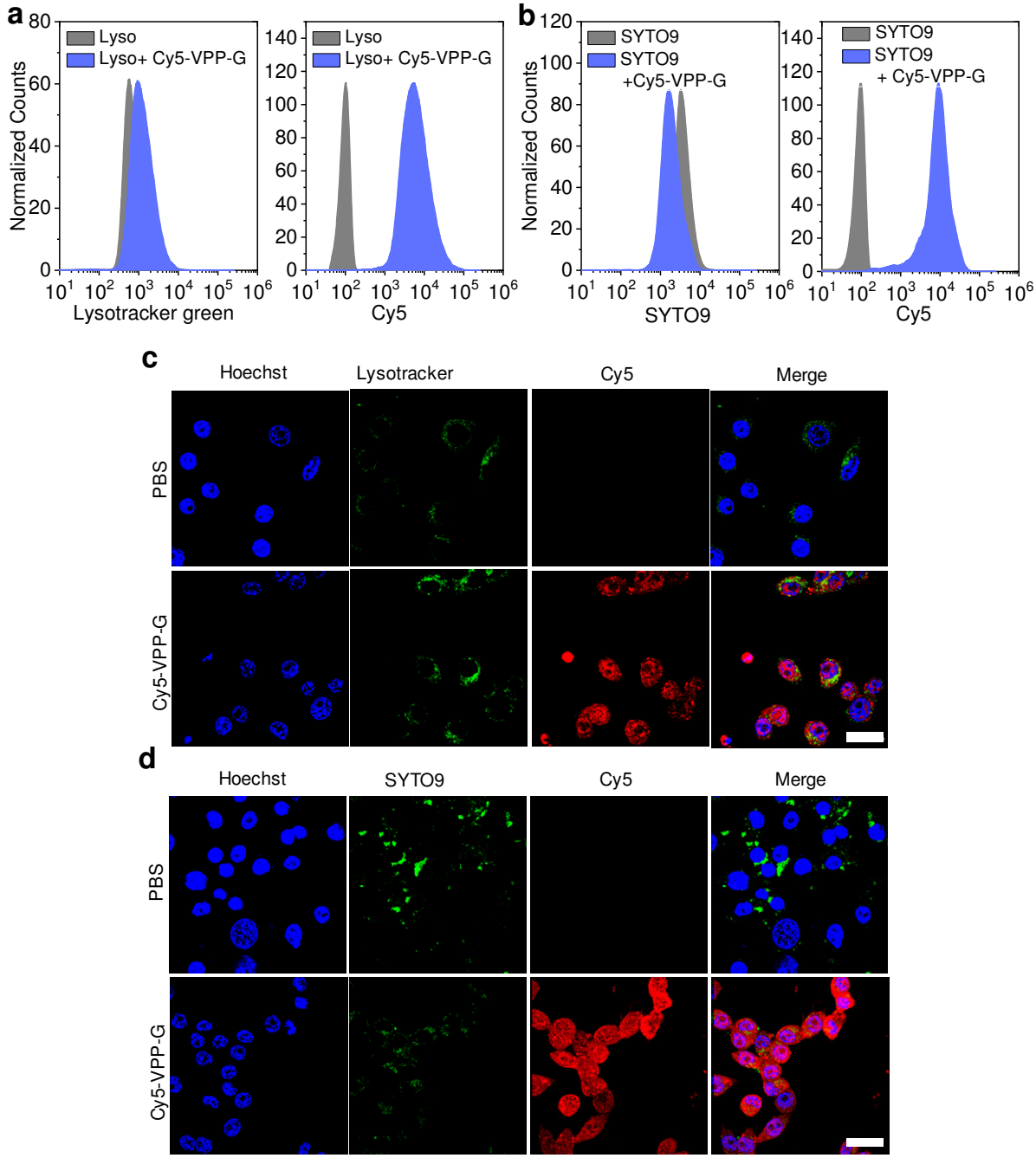


Figure S13. (a) Normalized counts of Lysotracker-stained RAW264.7 cells with or without Cy5-VPP-G treatment. Left: Lysotracker channel, right: Cy5 channel. (b) Normalized counts of *S. aureus* infected RAW264.7 cells with and without Cy5-VPP-G treatment. *S. aureus* was first stained with SYTO9 before infection. Left: SYTO9 channel, right: Cy5 channel. c) Confocal images of RAW264.7 macrophages stained with Lysotracker with and without Cy5-VPP10-G pretreatment. Blue: Hoechst, green: lysotracker, red: Cy5-VPP10-G. Scale bar: 20 µm. (d) Confocal images of *S.aureus* infected RAW264.7 cells treated with and without VPP10-G. *S.aureus* was stained with SYTO 9 before infection. Blue: Hoechst, green: SYTO9, red: Cy5-VPP10-G. Scale bar: 20 µm.

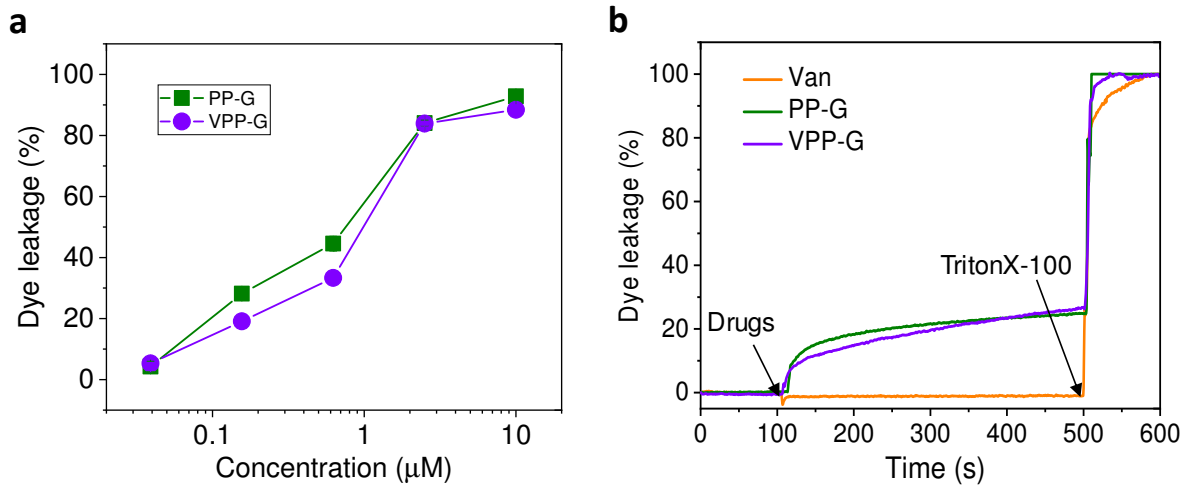


Figure S14. (a) Dye leakage profile from liposomes (DOPG/cardiolipin = 50/50) treated with VPP-G and PP-G at various concentrations. The fluorescent intensity was measured at t = 500 s after the addition of VPP-G or PP-G to liposome. (b) Dye leakage profile of mammanlian-mimicking liposome (DOPC/Cholesterol = 60/40) after treatment with VPP-G (9 μM), PP-G (9 μM) or vancomycin (9μM). Drugs were added at t ~ 100 s and 0.1% Triton X-100 was added at t ~ 500 s to completely lyse the liposomes.

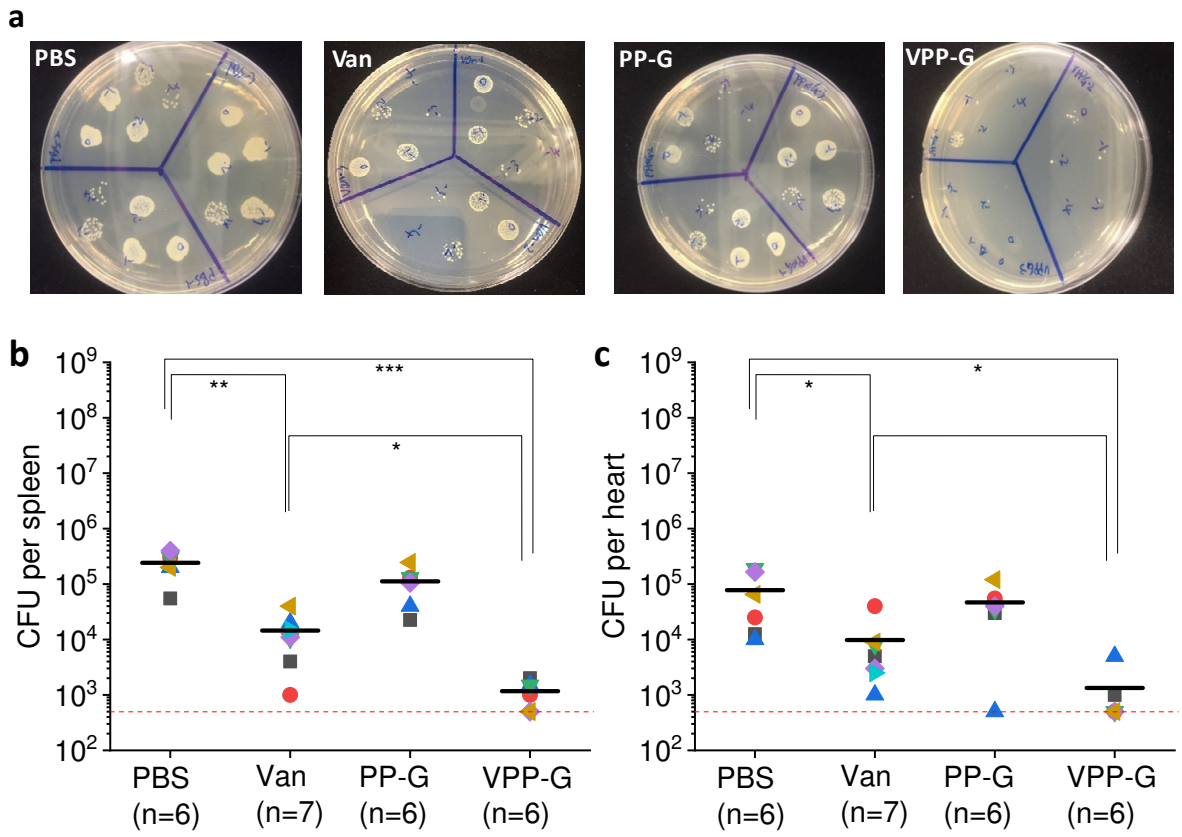


Figure S15. (a) Colonies recovered from the lysate of kidney of MRSA-infected mice treated with PBS, vancomycin, PP-G and VPP-G. 0, -1, -2, -3 and -4 represent 10^0, 10^1, 10^2, 10^3, and 10^4, dilutions, respectively. Bacteria burden of spleen (b) and heart (c) from mice treated with PBS, vancomycin (100 mg/kg), VPP-G (72 mg/kg, 1.5 × $MBC_{99.9}$) and PP-G (50 mg/kg, equivalent mole number as VPP-G). Dotted red line indicates detectoion limit. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Table S1: Kidney histopathological findings and scoring[a] by H&E staining.

Histopathological lesions	Uninfected	MRSA infected+ PBS	MRSA infected+ Van	MRSA infected+ PP-G	MRSA infected+ VPP-G
Intralesional bacterial colonies	1	5	1	1.5	1
Neutrophil infiltration	1	5	1	2.5	2
Tubular necrosis and degeneration	1	4	1	2	2

[a] Degree of lesions stained with H&E was graded from one to five depending on severity: 1 = minimal (< 1%); 2 = slight (1-25%); 3 = moderate (26-50%); 4 = moderate/severe (51-75%); 5 = severe/high (76-100%)

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