Supporting Information

Lipase Sensitive Polymeric Triple-Layered Nanogel for "On-Demand" Drug Delivery

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Materials and Reagents: 2-Chloro-2-oxo-1,3,2-dioxaphospholane (COP) was synthesized by a method described previously,[1] and distilled under reduced pressure before use. Triethylamine was refluxed with phthalic anhydride, then with potassium hydroxide, and distilled before use. Diethylene glycol (Sinopharm Chemical Reagent Co., Ltd., China) was dried over calcium hydride (CaH_2) overnight and distilled under reduced pressure before use. Toluene was refluxed over sodium, and tetrahydrofuran (THF) was dried over potassium-sodium and distilled just before use. Monomethoxy poly(ethylene glycol) with Mn=5000 (mPEG, Acros Organics) was dried twice by azeodistillation of anhydrous toluene. ε-Caprolactone (ε-CL; Acros Organics, 99%) was dried over CaH_2 for 48 h at room temperature, followed by distillation under reduced pressure just before use. Butytin was obtained from Alfa Co.

Characterization. Bruker AV300 NMR spectrometer was used for ^{1}H, ^{13}C and ^{31}P-NMR spectra measurements. Deuterated chloroform containing 0.03% (v/v) tetramethylsilane was used as the solvent. Phosphoric acid (85%) was used as the external reference for ^{31}P-NMR analyses.

The size and size distribution of the triple-layered nanogel (TLN) were measured by dynamic light scattering (DLS) carried out on a Malvern Zetasizer Nanao ZS90 with a He-Ne laser (633 nm) and 90° collecting optics. Sample was prepared at a concentration of 1 mg mL^{-1}. Measurements were carried out at 25 °C, and data were analyzed by Malvern Dispersion Technology Software 4.20.

Transmission electron microscopy was performed on JEM-2100 (JEOL Ltd., Japan) with an

accelerating voltage of 200 KV. The samples were prepared by pipetting a drop of the TLN in aqueous solution (0.2 mg mL^{-1}) onto a 230 mesh copper grids coated with carbon and allowing drying in air before measurements.

High-performance liquid chromatography (HPLC) analysis was performed using a Waters HPLC system consisting of Waters 1525 binary pump, Waters 2487 UV-vis detector, 1500 column heater and a Symmetry C18 column. HPLC grade acetonitrile and water with 0.1% (v/v) trifluoroacetic acid (12:88, v/v) was used as the mobile phase at 20 °C with a flow rate of 1.0 mL min^{-1}. Vancomycin was detected by a UV-vis detector at 280 nm and linked to Breeze software for data analysis. The concentration of vancomycin was acquired by comparing with a linear standard curve of vancomycin at different concentrations.

Spectrofluorophotometric analysis was performed on a Shimadzu RF-5301PC spectrofluorophotometer. The emission spectra were recorded at 20 °C with the detection wavelength λ_{ex} at 535 nm and a slit width of 5 nm. The concentration of propidium iodide (PI) was acquired by comparing with a linear standard curve of PI at different concentration.

Synthesis of 3-oxapentane-1,5-diyl bis(ethylene phosphate) (DEGDP). Under magnetic stirring at -5 °C, to a solution of COP (8.06 g, 0.06 mol) in 200 mL of anhydrous THF was added dropwise a mixture of diethylene glycol (3.00 g, 0.03 mol) and triethylamine (5.73 g, 0.06 mol) in 100 mL of anhydrous THF over a period of 2 h. The mixture was maintained at -5 °C for additional 4 h. The triethylammonium chloride precipitate was filtered off using a Schlenk funnel containing dried silica gel. The filtrate was concentrated under vacuum, washed with 50 mL of toluene twice, and dried under vacuum over night to obtain the product DEGDP with a yield of 28.5%. The structure of DEGDP was confirmed by ^{1}H and ^{13}C-NMR as shown in Figure S1.

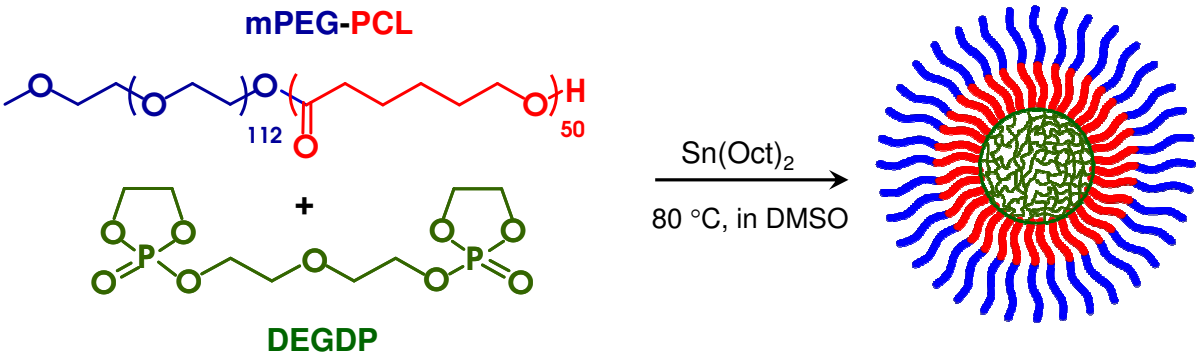
^{1}H-NMR (in $CDCl_3$, ppm): 4.36 (m, 8H, -OCH_2CH_2OP-), 4.15 (m, 4H, -POC$H_2$$CH_2O$-$CH_2CH_2$O-P-), 3.67 (t, 4H, -POC$H_2CH_2$O-C$H_2$$CH_2O$-P-); ^{13}C-NMR (in $CDCl_3$, ppm): 64.66 (-OCH_2CH_2OP-), 62.21 (-POC$H_2$$CH_2O$-$CH_2CH_2$O-P-), 60.91 (-POC$H_2CH_2$O-C$H_2$$CH_2O$-P-); ^{31}P-NMR (in $CDCl_3$, ppm): 12.87.

Synthesis of monomethoxy poly(ethylene glycol)-poly(ε-caprolactone) (mPEG-PCL). mPEG-PCL was synthesized by ring-opening polymerization of ε-caprolactone using mPEG as a macroinitiator and $Sn(Oct)_2$ as the catalyst. Briefly, ε-CL (12.723 g, 0.111 mol) and mPEG (11.100 g, 0.002 mol) were weighed into a round bottomed flask equipped with a magnetic stirring bar, and $Sn(Oct)_2$ (ca. 0.1% of ε-CL in molar amount) was added. The flask was sealed under dry argon and immersed in an oil bath at 120 °C for 24 h. The product was dissolved in dichloromethane, then purified by precipitation into cold diethyl ether. It was further vacuum-dried over night. The structure of mPEG-PCL was confirmed by ^{1}H and ^{13}C-NMR as shown in Figure S2. From the ^{1}H-NMR spectrum of the product, the degree of polymerization of PCL block was 50, calculated based on resonances at 3.66 ppm and 4.08 ppm assigned to methylene protons (-OC*H*$_2$C*H*$_2$O-) from mPEG and methylene protons (-OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_2$C(O)-) from PCL, respectively. The polydispersity of mPEG-PCL is 1.28, estimated by GPC measurement using polystyrene standards. The GPC system is composed of a Waters 1515 pump and a Waters 2414 refractive index detector equipped with Waters Styragel® High Resolution columns (1×HR4, 1×HR2 and 1×HR1, effective molecular-weight range 5 000-500 000, 500-20 000, 100-5 000, respectively). Chloroform was used as mobile phase at the flow rate of 1 mL min^{-1} at 40 °C. The molecular weight and molecular weight distribution were analyzed using Waters Breeze software. Monodispersed polystyrene standards were used to generate the calibration curve.

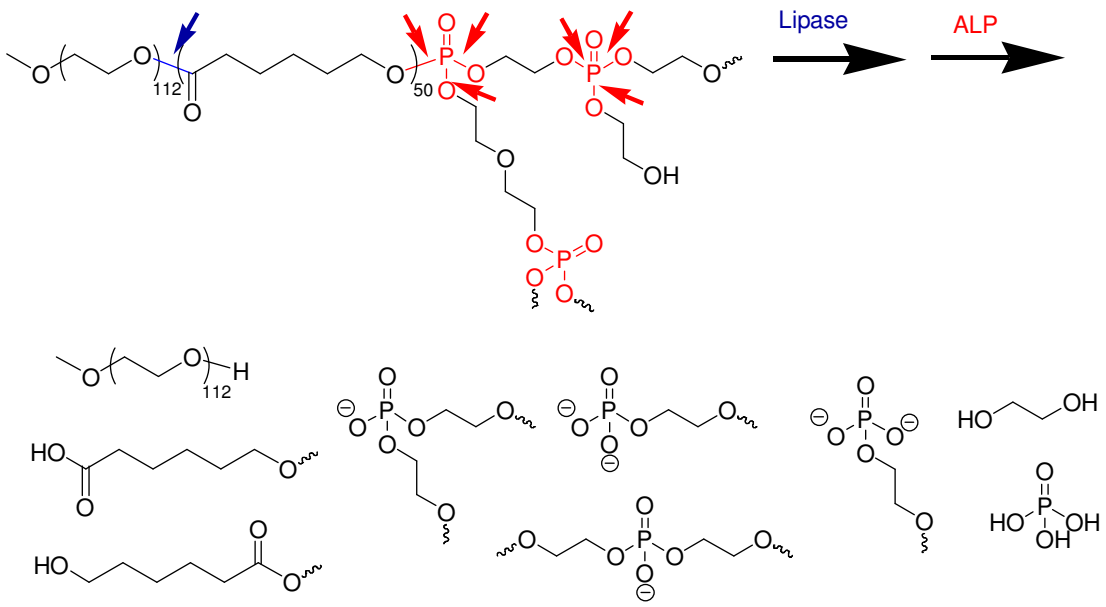
^{1}H-NMR (in $CDCl_3$, ppm): 4.08 (t, 2H, -OC*H*$_2$CH$_2$CH$_2$CH$_2$CH$_2$C(O)-), 3.66 (s, 4H, -OC*H*$_2$C*H*$_2$O-), 3.39 (s, 3H, C*H*$_3$-OCH$_2$CH$_2$O-), 2.32 (t, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$C*H*$_2$C(O)-), 1.66 (m, 4H, -OCH$_2$C*H*$_2$CH$_2$C*H*$_2$CH$_2$C(O)-), 1.40 (m, 2H, -OCH$_2$CH$_2$C*H*$_2$CH$_2$CH$_2$C(O)-)

Cytotoxicity assay. The relative cytotoxicity of the TLN, vancomycin-loaded TLN (TLN-V) or vancomycin was assessed with a methyl tetrazolium (MTT, Sigma-Aldrich Chemical Co.) viability assay against Raw264.7 cells or HEK293 cells. Sodium dodecyl sulfate (SDS) was used a control. The cells were seeded in 96-well plate at 10,000 cells per well in 100 μL of Dulbecco's Modified Eagle's

Medium culture medium containing 10% FBS, and incubated at 37 °C in 5% CO_2 humidified atmosphere for 24 hours. The medium was then replaced with 100 μL of TLN, TLN-V or vancomycin in complete culture medium at different concentrations. The cells were further incubated for 24 h, and then 25 μL of MTT stock solution (5 mg mL^{-1} in PBS) was added to each well to achieve a final concentration of 1 mg mL^{-1}, with the exception of the wells as blank, to which 25 μL of PBS was added. After incubation for another 2 h, 100 μL of extraction buffer (20% SDS in 50% DMF, pH 4.7, prepared at 37 °C) was added to the wells and incubated overnight at 37 °C. The absorbance was measured at 570 nm using a Bio-Rad 680 microplate reader. The cell viability was normalized to that of Raw264.7 cells cultured in complete culture medium.



Scheme S1. Synthesis scheme of the polymeric triple-layered nanogel (TLN) using mPEG-PCL to initiate the ring-opening polymerization of the bifunctional monomer DEGDP.



Scheme S2. Schematic illustration of enzyme-catalyzed degradation. The blue arrow indicates the lipase cleavage point, and the red arrows indicate the alkaline phosphatase (ALP) cleavage points.

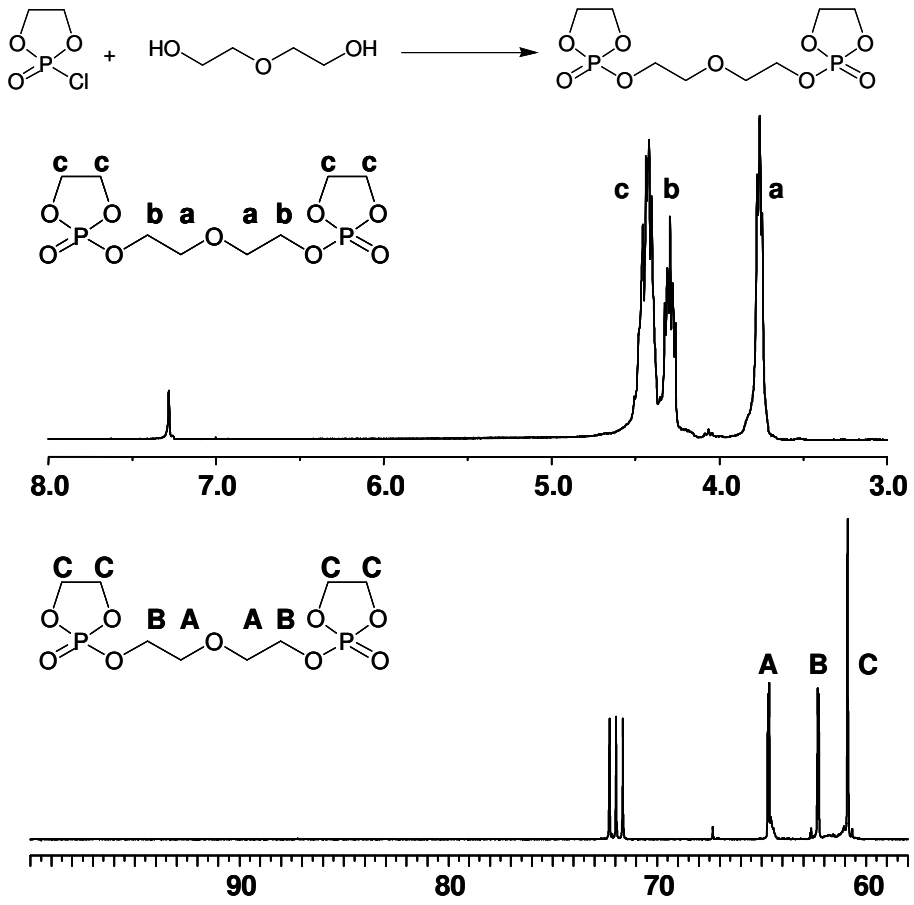


Figure S1. Synthesis scheme of monomer 3-oxapentane-1,5-diyl bis(ethylene phosphate) (DEGDP). ^{1}H and ^{13}C-NMR spectra of DEGDP (ppm).

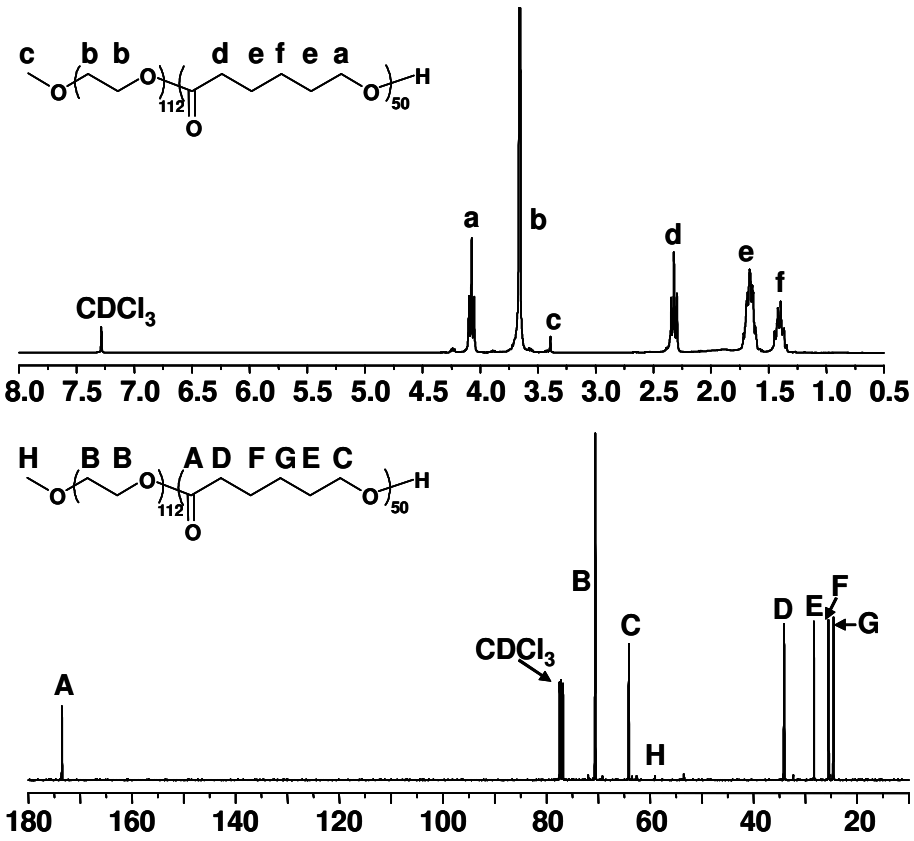


Figure S2. ^{1}H and ^{13}C-NMR spectra of macroinitiator monomethoxy poly(ethylene glycol)-poly(ε-caprolactone) (mPEG-PCL) (ppm).

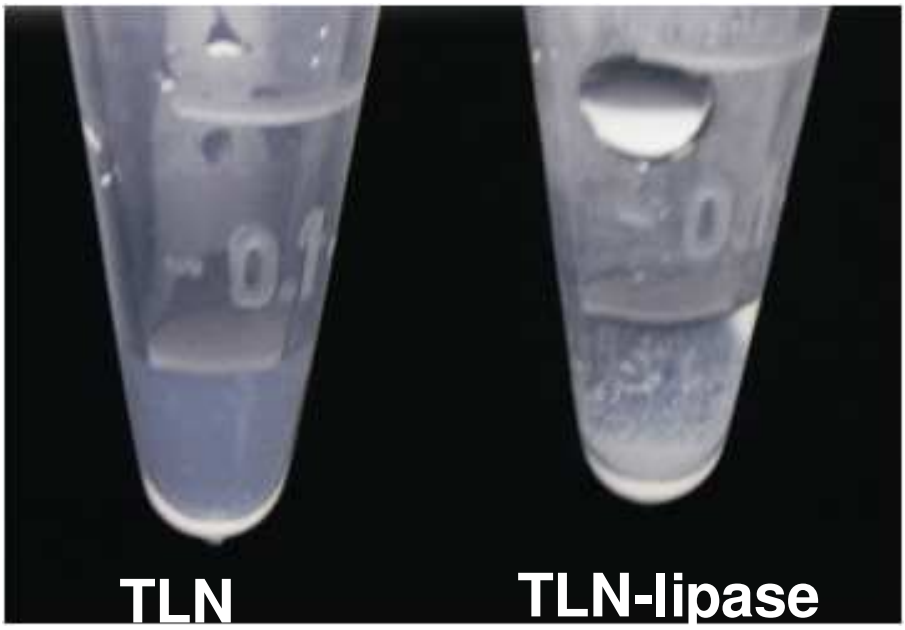


Figure S3. Photograph of aqueous solution of TLN (left) and lipase treated TLN (right).

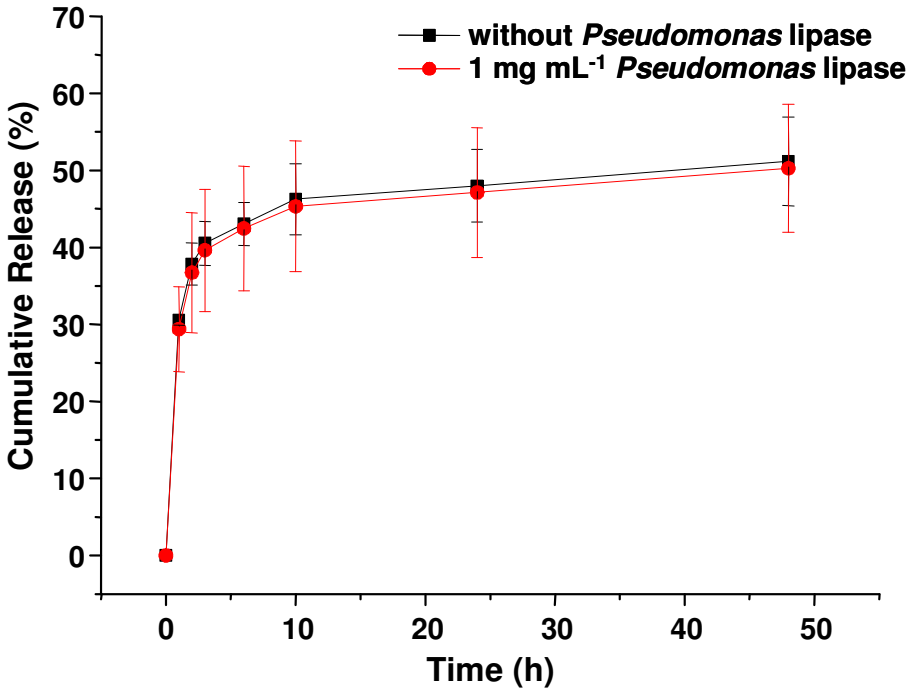


Figure S4. Cumulative release of vancomycin from vancomycin-loaded PEG-polyphosphoester (PEG-DEGDP) nanogel. Vancomycin-loaded PEG-DEGDP nanogel was cultured in Tris-HCl buffer (0.01M, pH 7.4) in the absence or presence of *Pseudomonas* lipase.

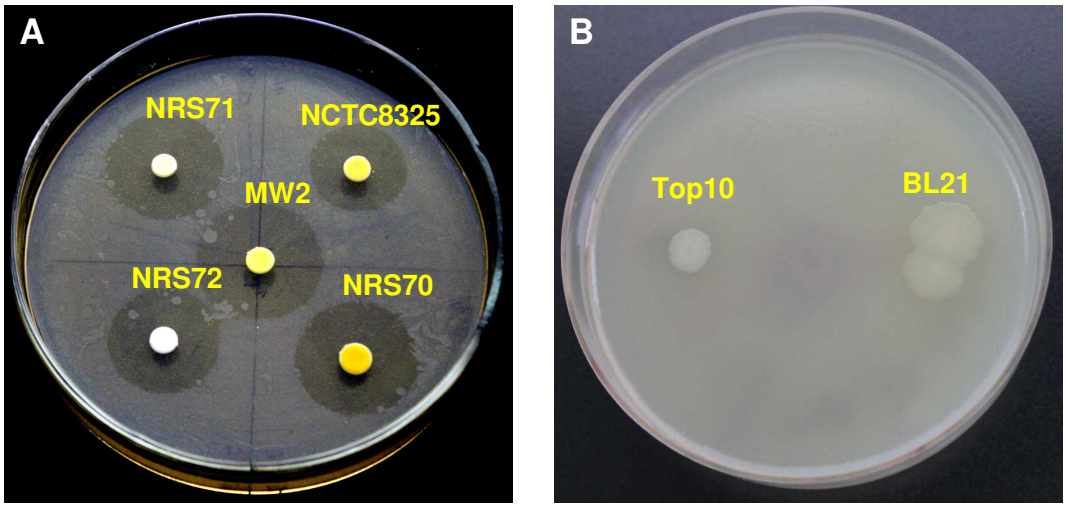


Figure S5. Tributyrin agar test of bacterial lipase. A drop of *S. aueus* strains, MW2, NCTC8325, NRS70, NRS71, NRS72, were plated on the culture dish with tributyrin agar (A). A drop of *Escherichia coli* (*E. Coli*) strains, Top10 and BL21, were plated on the culture dish with tributyrin agar (B). Tributyrin agar is a differential medium that tests the ability of lipase who hydrolyzes tributyrin oil. Lipase allows the organisms that produce it to break down lipids into smaller fragments. Tributyrin oil forms an opaque suspension in the agar. When an organism produces lipase and breaks down the tributyrin, a clear halo surrounds the areas where the lipase-producing organism has grown. It is suggested that lipase is secreting in all five *S. aueus* strains but not in *E. coli* strain Top10 and BL21.

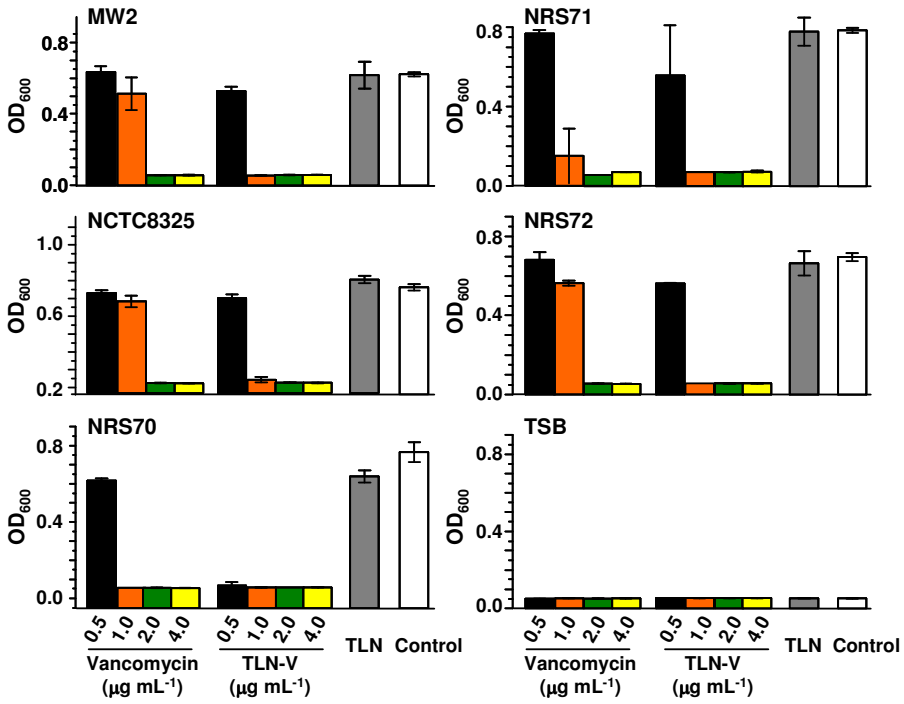


Figure S6. Dose-dependent growth inhibition of *S. aureus* strains at 24 h in the presence of free vancomycin, vancomycin-loaded TLN (TLN-V), or TLN (95.2 μg mL^{-1}, equal to the concentration of TLN in culture that the bacteria was treated with TLN-V at a vancomycin concentration of 4 μg mL^{-1}). The concentration given in Vancomycin or TLN-V treatment represents the final concentration of vancomycin in the culture. The data are expressed as mean ± standard deviation of three replicates. Standard deviation is indicated by the error bars. The control is bacteria in Bacto™ Tryptic Soy Broth (TSB).

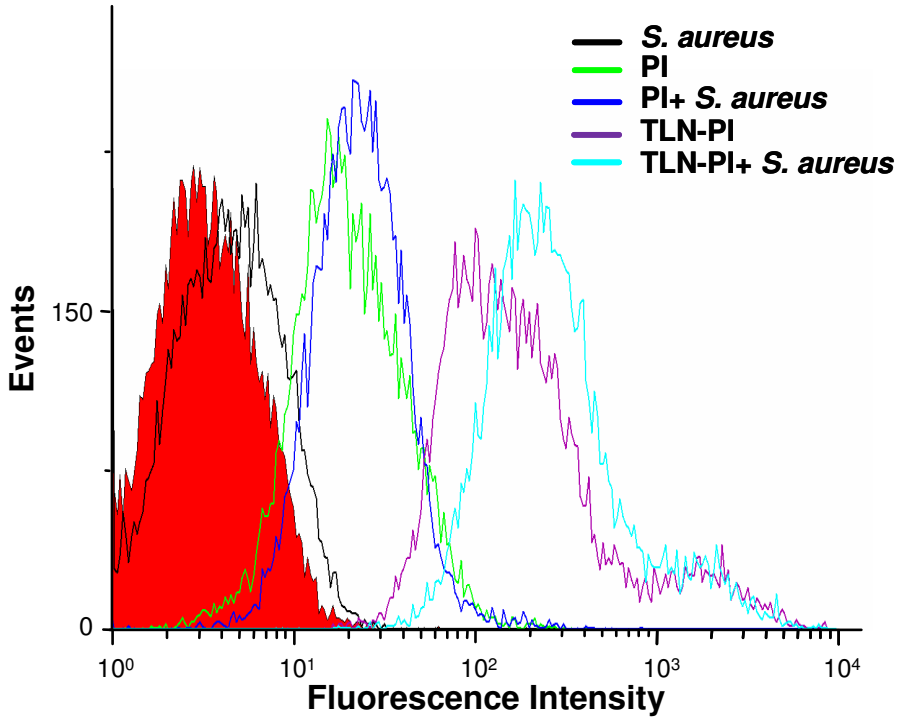


Figure S7. Cellular uptake of propidium iodide (PI)-loaded TLN (TLN-PI) and PI in the Raw264.7 cells or cells infected by *S. aureus* strain NCTC8325 as analyzed by flow cytometric analyses at 10 h. For the infection studies, cells were first infected with bacteria. The multiplicity of infection was 10 bacteria per cell. Cells were incubated with the processed bacteria for 1 h to allow for phagocytosis. The media were then replaced with fresh media containing 1 μg mL^{-1} lysostaphin, and the infected cells were further incubated for 30 min to eliminate extracellular bacteria. Then TLN-PI (1 μL, 10 mg mL^{-1} in water) or PI was added. After incubation at 37 °C for 10 h, cells were trypsinized, washed with PBS twice, resuspended in 200 μL of PBS and subjected to flow cytometric analysis.

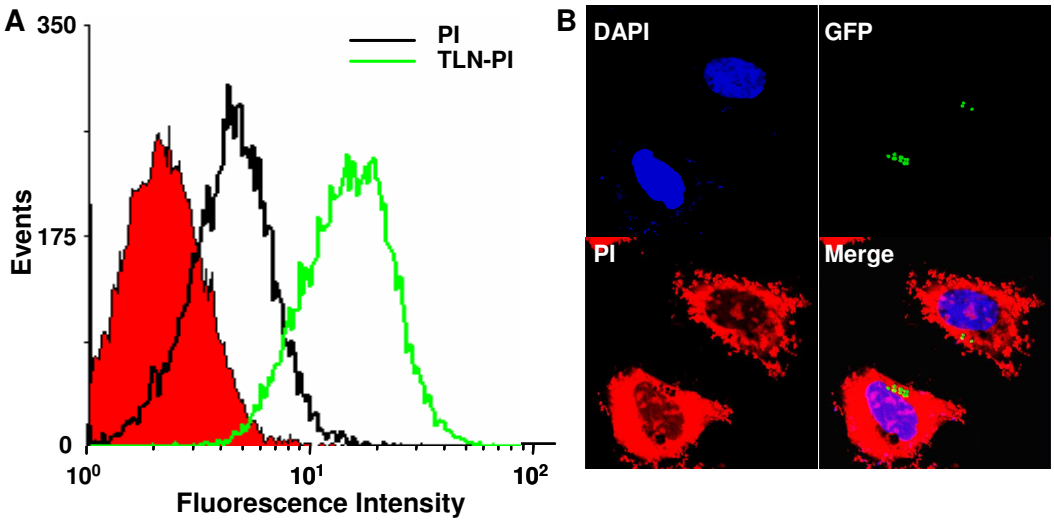


Figure S8. Cellular uptake of TLN-PI and PI at 4 h as analyzed by flow cytometric analyses (A), and the internalization of TLN-PI by HEK293 cells infected with the GFP-expressing strain NCTC8325 (B).

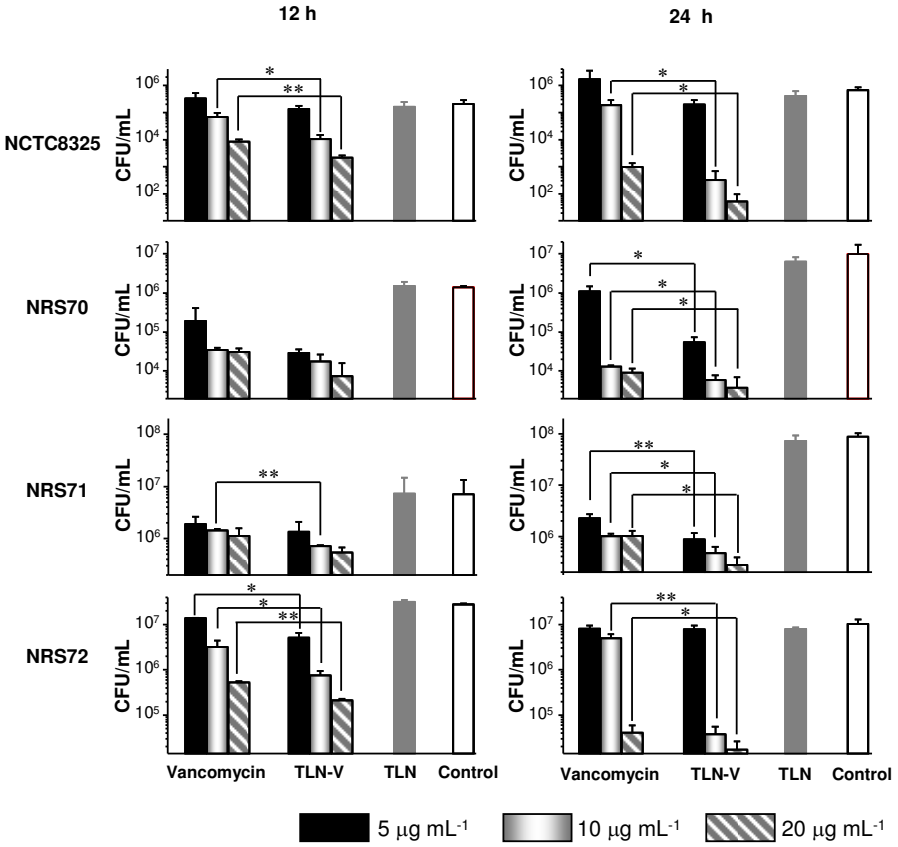


Figure S9. Intracellular survival of *S. aureus* in Raw264.7 cells. Infected cells were cultured with vancomycin, vancomycin-loaded TLN (TLN-V), empty TLN (476.2 μg mL^{-1}, equal to the concentration of TLN in culture that the cells were treated with TLN-V at a vancomycin concentration of 20 μg mL^{-1}) or left untreated (control). The final concentration of vancomycin in the culture was 5, 10 or 20 μg mL^{-1} when it was applied. The incubation was terminated after 12 h or 24 h to determine the intracellular survival of *S. aureus*. CFU, colony-forming units. ∗ represents $p < 0.05$, and ∗∗ represents $p < 0.01$ determined by Student's *t* test.

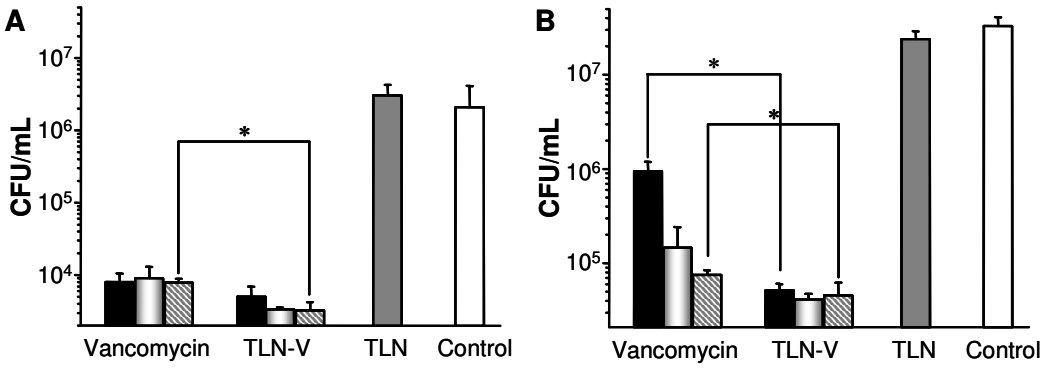


Figure S10. Intracellular survival of *S. aureus* MW2 in HEK293 cells. Infected cells were cultured with vancomycin, TLN-V, empty TLN (476.2 μg mL^{-1}, equal to the concentration of TLN in culture that the cells were treated with TLN-V at a vancomycin concentration of 20 μg mL^{-1}) or left untreated (control). The final concentration of vancomycin in the culture was 5 (■), 10 (▤) or 20 (▨) μg mL^{-1} when it was applied. The incubation was terminated after 12 h (A) or 24 h (B) to determine the intracellular survival of *S. aureus*. CFU, colony-forming units. ∗ represents $p < 0.05$ determined by Student's t test.

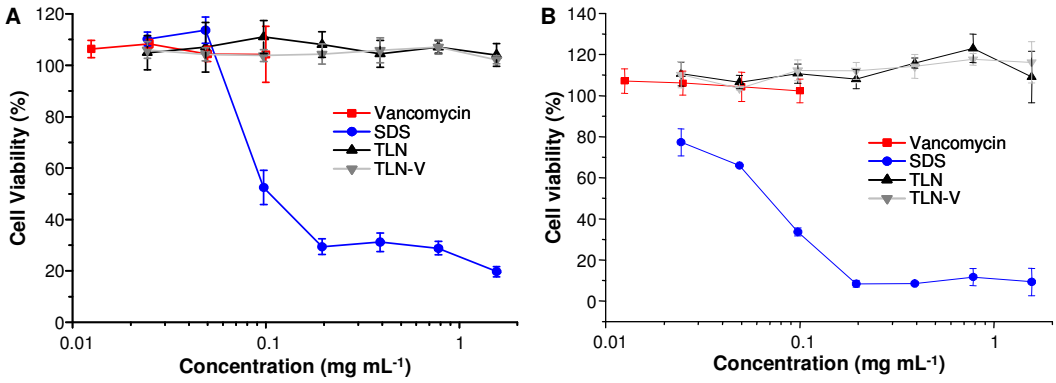


Figure S11. Cytotoxicity of TLN, TLN-V and vancomycin to Raw264.7 cells (A) and HEK293 cells (B) in comparison with SDS after 24 h incubation. The data are expressed as mean ± standard deviation of three replicates.

[1] R. S. Edmundson, *Chemistry Industry* **1962**, 1828.

Reference 2:

C. E. Ashley, E. C. Carnes, G. K. Phillips, D. Padilla, P. N. Durfee, P. A. Brown, T. N. Hanna, J. W. Liu, B. Phillips, M. B. Carter, N. J. Carroll, X. M. Jiang, D. R. Dunphy, C. L. Willman, D. N. Petsev, D. G. Evans, A. N. Parikh, B. Chackerian, W. Wharton, D. S. Peabody, C. J. Brinker, *Nat. Mater.* **2011**, *10*, 389-397.