Supporting information for:

Degradable polyphosphoester-based silver-loaded nanoparticles as therapeutics for bacterial lung infections

Fuwu Zhang,[a] Justin A. Smolen,[b] Shiyi Zhang,[a] Richen Li,[a] Parth N. Shah,[b] Sangho Cho,[a] Hai Wang,[a] Jeffery E. Raymond,[a] Carolyn L. Cannon[b,*] and Karen L. Wooley[a,*]

[a]*Department of Chemistry, Department of Chemical Engineering, and Department of Materials Science & Engineering, Laboratory for Synthetic- Biologic Interactions, Texas A&M University, P.O. Box 30012, 3255 TAMU, College Station, Texas 77842-3012, United States, Fax: +1 979 862 1137;Tel.: +1 979 845 4077; E-mail: wooley @chem.tamu.edu*

[b]*Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M Health Science Center, 407 Reynolds Medical Building, College Station, TX 77843-1114, United States, E-mail: cannon@medicine.tamhsc.edu*

EXPERIMENTAL SECTION

Materials. 2-chloro-2-oxo-1,3,2-dioxaphospholane (95%) was used as received from Thermo Fisher Scientific Inc. (Pittsburgh, PA). Chelex 100 Resin was used as received from Bio-Rad Laboratories (Hercules, CA). Tetrahydrofuran (THF) and dichloromethane (DCM) were dried through columns (J. C. Meyer Solvent Systems, Inc., Laguna Beach, CA). α-methoxy-ω-azido PEG_{2k} was purchased from RAPP POLYMERE (Tuebingen, Germany). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification unless otherwise noted. Slide-A-Lyzer dialysis cassettes (10 kDa molecular weight cut-off, MWCO) were purchased from Pierce Biotech. (Rockford, IL). The Spectra/Por dialysis membranes (MWCO 12-14 kDa) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Nanopure water (18 MΩ·cm) was acquired by means of a Milli-Q water filtration system, Millipore Corp. (Bedford, MA). Dr. Maynard Olson (University of Washington, Seattle, WA) provided the laboratory strain PAO1-V, and Dr. Thomas Ferkol (Washington University, St. Louis, MO) provided the mucoid, cystic fibrosis clinical isolate PAM57-15. PAHP3, BG80, BM54, SALL06, and SAEH05 were cultured and isolated from cystic fibrosis patient sputum at St. Louis Children's Hospital (St. Louis, MO). The USA300 strain of methicillin-resistant *S. aureus*, TCH1516, was acquired from ATCC (BAA-1717).

Instrumentation. ^{1}H NMR and ^{13}C NMR spectra were recorded on Inova 300 spectrometer interfaced to a UNIX computer using VnmrJ software. Chemical shifts were referenced to the solvent residual signals.

FTIR spectra were recorded on an IR Prestige 21 system using a diamond ATR lens (Shimadzu Corp., Japan) and analyzed using IRsolution v. 1.40 software.

The DMF gel permeation chromatography (GPC) was conducted on a Waters Chromatography, Inc. (Milford, MA) system equipped with an isocratic pump model 1515, a differential refractometer model 2414, and a three-column set of Styragel HR 4 5 μm DMF (300 × 7.5 mm), Styragel HR 4E 5 μm DMF (300 × 7.5 mm), and Styragel HR 2 5 μm DMF (300 × 7.5 mm). The system was equilibrated at 70 °C in pre-filtered DMF containing 0.05 M LiBr, which served as polymer solvent and eluent (flow rate set to 1.00 mL/min). Polymer solutions were prepared at a concentration of *ca*. 3 mg/mL and an injection volume of 200 μL was used. Data collection and analysis were performed with Empower 2 v. 6.10.01.00 software (Waters, Inc.). The system was calibrated with polystyrene standards (Polymer Laboratories, Amherst, MA) ranging from 615 to 442,800 Da.

Dynamic light scattering (DLS) measurements were conducted using a Delsa Nano C (Beckman Coulter, Inc., Fullerton, CA) instrument equipped with a laser diode operating at 658 nm. Size measurements were made in nanopure water ($n = 1.3329$, $\eta = 0.890$ cP at 25 ± 1 °C). Scattered light was detected at 165° angle and analyzed using a log correlator over 70 accumulations for a 3.0 mL sample in a glass sizing cell (4.0 mL capacity). The photomultiplier aperture and the attenuator were automatically adjusted to obtain a photon counting rate of *ca*. 8 kcps. Calculation of the particle size distribution and distribution averages was performed using CONTIN particle size distribution analysis routines. The peak averages of histograms from number distributions out of 70 accumulations were reported as the average diameters of the particles. The particle ζ-potentials were determined by a Delsa Nano C particle analyzer (Beckman Coulter, Fullerton, CA) equipped with a 30 mW dual laser diode (658 nm). The ζ-potential of the particles in suspension was obtained by measuring the electrophoretic movement of charged particles under an applied electric field. Scattered light

was detected at a 30° angle at 25 °C. The ζ-potential was measured at five regions in the flow cell, and a weighted mean was calculated. These five measurements were used to correct for electro-osmotic flow that was induced in the cell due to the surface charge of the cell wall. All determinations were repeated five times.

Glass transition temperatures (T_g) were measured by differential scanning calorimetry on a Mettler-Toledo DSC822® (Mettler-Toledo, Inc., Columbus, OH), with a heating rate of 10 °C/min. Measurements were analyzed using Mettler-Toledo Star^e v. 7.01 software. The T_g was taken as the midpoint of the inflection tangent, upon the third heating scan. Thermogravimetric analysis was performed under N_2 atmosphere using a Mettler-Toledo model TGA/SDTA851^e, with a heating rate of 10 °C /min. Measurements were analyzed by using Mettler-Toledo Stare v. 7.01 software.

Transmission electron microscopy (TEM) images were collected on a JEOL 1200EX operating at 100 kV and micrographs were recorded at calibrated magnifications using a SIA-15C CCD camera. The samples as aqueous solutions (4 μL) were deposited onto carbon-coated copper grids, and after 1 min, the excess of the solution was quickly wicked away by a piece of filter paper. A drop of 1 *wt*% uranyl acetate was then added, and allowed to stand for 30 seconds before excess stain was wicked away. The grids were allowed to dry in air overnight. High-resolution scanning transmission electron (STEM) microscopy and elemental mapping were conducted on a FEI Tecnai G2 F20 FE-TEM coupled with energy-dispersive X-ray (EDX), operating at a voltage of 200 kV with a Gatan CCD camera.

The atomic force microscopy (AFM) imaging was performed on MFP-3D system (Asylum Research) in tapping mode using standard silicon tips (VISTAprobes, CSR-25, resonance constant: 28 kHz, tip radius: <10 nm, spring constant: 0.1 N/m). For AFM sample preparation,

nanoparticles were dissolved in nanopure water at 0.025 mg/mL, and 10 μL of the sample was spin-coated onto a mica surface and allowed to dry in air overnight.

A B&W Tek iRaman system (785 nm, 300 mW max) was used to assess the polyphosphoester system upon association with silver. Measurements were obtained through a 20x objective (PLL, Olympus) at 30 mW with 1 s integration averaged over 10 spectra. Relative populations for the species in the acetylide fingerprint region were assessed by peak deconvolution using Origin 9.0 Pro software package.

Synthesis of EBP and BYP monomers. 2-(but-3-yn-1-yloxy)-2-oxo-1,3,2-dioxaphospholane (BYP) and 2-(2-ethylbutoxy)-1,3,2-dioxaphospholane (EBP) were synthesized as previously described.[1]

Synthesis of PEBP-*b*-PBYP diblock copolymer. A solution of EBP (1.5 g, 7.2 mmol) and benzyl alcohol (15.6 mg, 0.14 mmol) in 2.1 mL anhydrous dichloromethane was transferred *via* syringe into a flame-dried vial equipped with a stir bar and rubber septum in an water/ice bath. A solution of 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) (40.2 mg, 0.29 mmol) in 0.3 mL anhydrous dichloromethane was injected quickly into the vial. After being stirred for 2 min, another 2.4 mL of anhydrous dichloromethane was injected into the vial to dilute the reaction mixture, and the vial was transferred to a cooling bath composed of equal amounts of ice and sodium chloride at -10 °C. Less than 0.1 mL of the reaction mixture was withdrawn and diluted with $CDCl_3$ to determine the EBP conversion by ^{31}P NMR. After stirring for another 2 min, the reaction mixture was augmented with a solution of BYP (1.27 g, 7.2 mmol) in 2.4 mL anhydrous dichloromethane *via* syringe. The reaction was quenched by adding an excess amount of acetic acid dissolved in DCM after another 3 min, and an aliquot of the mixture was used to determine the conversion of BYP by ^{31}P NMR. The PEBP-*b*-PBYP was purified by precipitation from

acetone into a pentane/diethyl ether mixture (3:1 volume ratio) three times and then dried under vacuum. ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 0.89 (t, J = 7.2 Hz, $POCH_2CH(CH_2CH_3)_2$), 1.37 (m, $POCH_2CH(CH_2CH_3)_2$), 1.50 (m, $POCH_2CH$), 2.11 (s, $POCH_2CH_2C{\equiv}CH$), 2.62 (m, $POCH_2CH_2C{\equiv}CH$), 4.00 (t, J = 5.6 Hz, $POCH_2CH$), 4.12–4.39 (m, $POCH_2CH_2OP$, $POCH_2CH_2$), 5.08 (d, J = 8.3 Hz OCH_2Ar), 7.32–7.41 (m, Ar-*H*); ^{13}C NMR (75 MHz, $CDCl_3$, ppm): 10.99, 20.69, 22.75, 41.55, 65.52-67.11, 70.08, 70.88, 79.59; ^{31}P NMR (121 MHz, $CDCl_3$, ppm): δ -1.25, -1.81. GPC: M_n = 25200 g/mol, PDI = 1.25. DSC: T_g = -59°C, - 45 °C. TGA in N_2: 180-365 °C, 58% mass loss; 365–500 °C, 6.1% mass loss, 35% mass remaining above 500 °C. IR (cm^{-1}): 3700-3150, 3000-2800, 1645, 1464, 1379, 1267, 1009, 966, 868, 806.

Azide-alkyne Huisgen cycloaddition of PEBP-*b*-PBYP with α-methoxy-ω-azido PEG (CH_3O-PEG_{2k}-azido). In a typical experiment, a dried vial containing a magnetic stir bar was charged with PEBP-*b*-PBYP (0.83 g, 43 μmol, 1 eq.), α-methoxy-ω-azido PEG (0.33 g, 0.17 mmol, 4 eq.), *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine (PMDETA, 12 mg, 69 μmol, 1.6 eq.) and 7 mL of DMF. The reaction mixture was degassed by several freeze-pump-thaw cycles (N>3), during which copper(I) bromide (4.9 mg, 34 μmol, 0.8 eq.) was added. The flask was allowed to return to room temperature after the final cycle and stirred for another 4 h. The solution was subsequently filtered through a neutral alumina column and dialyzed against Chelex 100 resin in nanopure water in presoaked dialysis tubing (MWCO.*ca*. 12–14 kDa) for 2 d to remove copper ions, followed by lyophilization to yield white powder with a 70 % yield. Inductively coupled plasma-mass spectrometry (ICP-MS) confirmed that less than 10 ppm of copper was present in the polymer. ^{1}H NMR (300 Hz, $CDCl_3$, ppm): δ 0.90 (t, J = 7.5 Hz, $POCH_2CH(CH_2CH_3)_2$), 1.37 (m, $POCH_2CH(CH_2CH_3)_2$), 1.50 (m, $POCH_2CH$), 2.11 (s, $POCH_2CH_2C{\equiv}CH$), 2.56-2.65 (m, $POCH_2CH_2C{\equiv}CH$), 3.12 (m, NCH_2CH_2), 3.38 (s, OCH_3), 3.64

(s, OCH_2CH_2), 4.00 (t, J = 5.7 Hz, POCH_2CH), 4.14-4.39 (m, POCH_2CH$_2$, POCH_2CH_2OP), 5.08 (d, J = 8.4 Hz, OCH_2Ar), 7.31-7.41 (m, Ar-H), 7.60-7.66 (m, CH$_2$CH$_2$CCHNCH$_2$). ^{13}C NMR (75 MHz, CDCl$_3$, ppm): δ 11.03, 20.72, 22.79, 41.60, 65.52-67.15, 70.13, 70.68, 70.95, 79.72, 128.06, 128.73. ^{31}P NMR (121 MHz, CDCl$_3$, ppm): δ -0.99, -1.64. GPC: M_n = 31300 g/mol, PDI = 1.26. DSC: T_g = - 52 °C; T_m = 42 °C. TGA in N$_2$: 180–265 °C, 41% mass loss; 265–380 °C, 32% mass loss, 360–600 °C, 1% mass loss, 26 % mass remaining above 600 °C. IR (cm^{-1}): 3800-3050, 3050-2700, 1651, 1460, 1348, 1271, 1016, 964, 866, 803.

Silver loading into micelles. In a typical experiment, PEBP-*b*-PBYP-*g*-PEG (32.0 mg, 1.0 eq.) was dissolved in 3.2 mL of nanopure water and sonicated for 3 min. Silver acetate (9.0 mg, 46 eq.) in 2.0 mL of nanopure water was added, and the mixture solution was shaded with aluminum foils and stirred overnight. The solution was transferred to a centrifugal filter device (100 kDa MWCO), and washed extensively for several cycles (N>3) with nanopure water to remove free small molecules. The amount of silver loaded into the micelles was quantified by ICP-MS using rhodium as an internal standard.

Release of silver from silver-loaded micelles. The release profiles of the silver-loaded micelles were studied by monitoring the decrease of silver concentration over time in dialysis cassettes by ICP-MS. In a typical procedure, 3.0 mL of silver-loaded micelles was transferred into a presoaked dialysis cassette. The cassette was allowed to stir in a beaker containing 3000 mL nanopure water or phosphate buffer (10 mM phosphate, 10 mM NaCl, pH 7.4) at 37 °C. Aliquots (*ca.* 0.05 mL) were taken at pre-determined time and silver concentrations were determined by ICP-MS. The release experiments were conducted in a triplicate manner.

Determination of Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs). The bacterial strains were streaked from frozen stocks

onto tryptic soy agar (TSA) plates and incubated at 37°C for 24-48 hours, depending on the growth rate of the strain. Single colonies were then inoculated into Mueller Hinton broth (MHB) and incubated at 37°C with 200 rpm shaking until they reached an optical density of 0.4 at 650 nm (OD_{650}), corresponding to approximately 5 x 10^8 CFU/mL. The silver-loaded micelles and silver acetate were suspended/dissolved and diluted in molecular-grade water and added to a 96-well plate (100 µL/well). The bacteria suspensions were diluted 1:500 in double-strength (2X) MHB and added 1:1 by volume to the diluted silver acetate or nanoparticles (10^5 CFU/well). The plates were then sealed with gas-permeable membranes (Breathe-Easy®) and incubated for 18-24 hours at 37°C. MICs were recorded as the lowest concentration of silver that yielded clear replicate wells. To determine MBCs, 100 µL of the contents of each clear well was dispensed onto TSA plates supplemented with 5% sheep blood. The plates were incubated 24-48 hours at 37°C, depending on the strain, and the MBCs were recorded as the lowest concentration of silver that yielded no growth. MIC/MBCs were all performed three times and the results with the highest MIC or MBC amongst the replicates were reported.

Cytotoxicity with AlamarBlue®. 16HBEs, provided by Dr. D. Gruenert (University of California, San Fransisco, CA), are a human bronchial epithelial cell line that were transformed with SV40 large T-antigen using the replication-defective pSVori plasmid. 16HBEs between passage 20 to 40 were cultured in Minimum Essential Medium with Earle's Balanced Salts with non-essential amino acids and supplemented with penicillin-streptomycin (1%), l-glutamine (1%), and fetal bovine serum (10%). Prior to cytotoxicity studies, the cells were lifted with trypsin, seeded in a 96-well plate (25,000 cells/well), and incubated overnight at 37°C, 5% CO_2, and 100% RH. The silver-loaded micelles and silver acetate were suspended/dissolved and diluted in the same feeding media and added fresh to the cells. After a 24 hour incubation, the

media with silver was removed and replaced with OPTI-MEM containing 10% AlamarBlue® (Life Technologies Inc.) and the plates were incubated for an additional 6 hours. To determine cytotoxicity, the plates were read with a plate-reading spectrophotometer (BMG Labtech) at 570 nm and 600 nm and cell viability was calculated according to manufacturer's instructions. IC_{50}s, based on molar concentration of silver, were calculated by generating a non-linear curve fit and interpolating the concentration yielding 50% inhibition (Graphpad Prism®). Viability assays were performed three times and averaged. Significant difference between the silver-loaded micelles and silver acetate was performed using non-parametric Student's t-test (Graphpad Prism®).

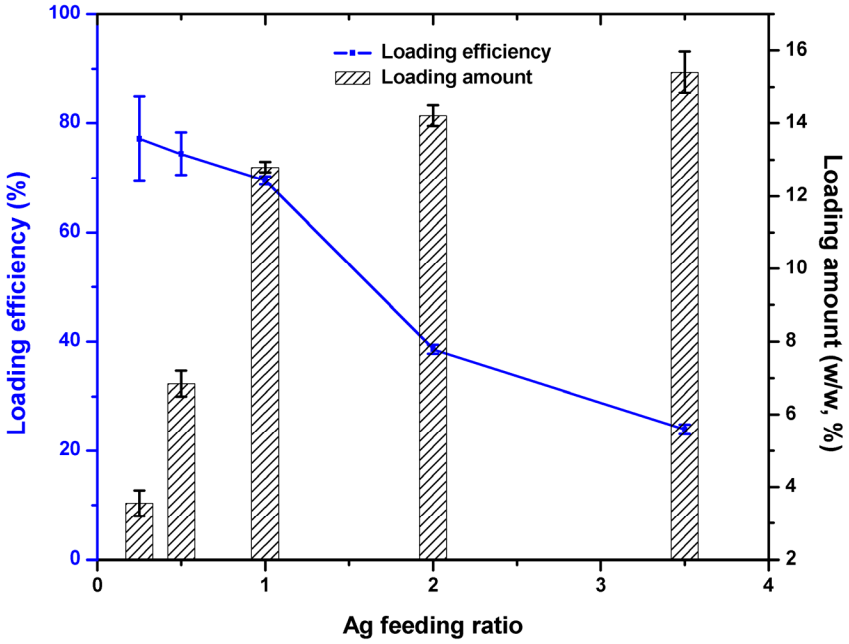

Fig. S1 Optimization of loading efficiency (left, blue line) and loading amount (right, gray bars). The experiment was done in triplicate.

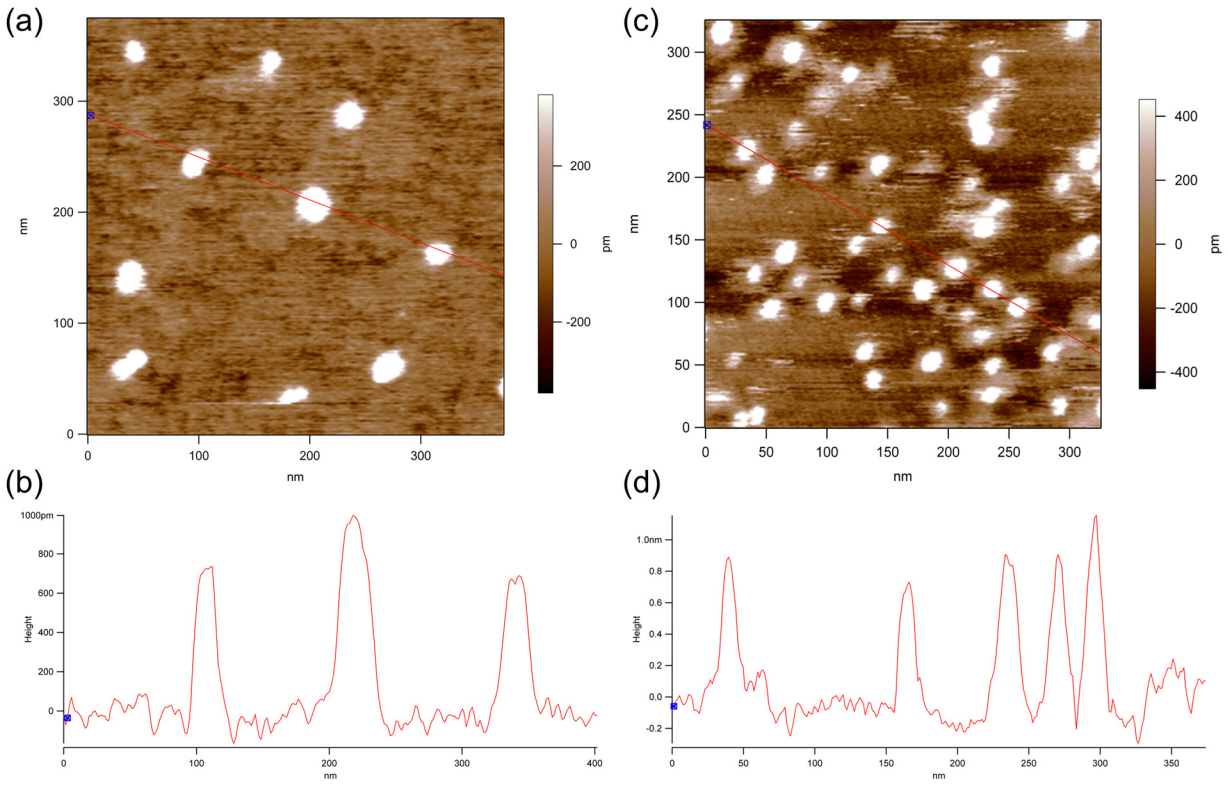

Fig. S2 (a) Tapping mode AFM height image of empty micelles, and (b) height profile along the red line drawn in (a), (c) Tapping mode AFM height image of silver-loaded nanoparticles and (d) height profile along the red line drawn in (c).

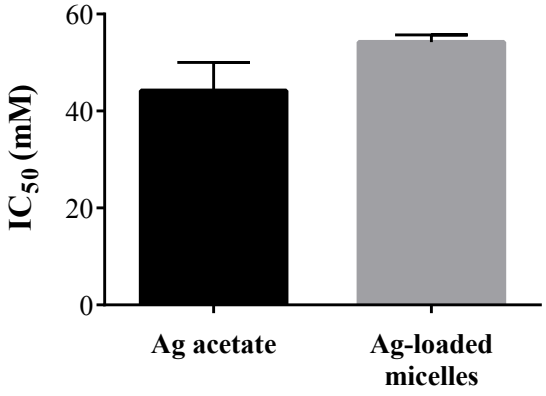

Fig. S3 Half-maximal inhibitory concentration (IC_{50}) of silver acetate and silver-loaded micelles against 16HBE cells, as determined by AlamarBlue®. Not significantly different (P = 0.25) according to non-parametric Student's t-test.

(1) Zhang, S.; Zou, J.; Zhang, F.; Elsabahy, M.; Felder, S. E.; Zhu, J.; Pochan, D. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2012**, *134*, 18467.