This journal is © The Royal Society of Chemistry 2013

SUPPORTING INFORMATION

Integrated Approach to Evaluating the Toxicity of Novel Cysteine-Capped Silver Nanoparticles to *Escherichia coli* and *Pseudomonas aeruginosa*

John H. Priester[1,2,3], Aditi Singhal[3,4], Binghui Wu[4], Galen D. Stucky[3,4,5] and Patricia A. Holden[1,2,3]*

[1]Bren School of Environmental Science & Management, [2]Earth Research Institute, [3]UC Center for the Environmental Implications of Nanotechnology, [4]Department of Chemistry and Biochemistry, [5]Materials Department, University of California, Santa Barbara, CA 93106

*Corresponding Author (holden@bren.ucsb.edu)

Table of Contents

1. Materials and Methods……………………………………………….. …….. 2
2. Supporting Tables…………………………………………………………. 4
3. Supporting Figures………………………………………………………… 5

This journal is © The Royal Society of Chemistry 2013

1. MATERIALS AND METHODS

Nano-Ag Characterization

UV-Vis spectrometry was performed using an Agilent 8453 UV-VIS-NIR Photodiode Array System (Agilent Technologies, Santa Clara, CA, USA), capable of measuring the entire UV-Vis spectral response in ~ 1 second. FT-IR was performed using a Nicolet Magna 850 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA) in mid-IR range. XRD powder diffraction pattern was obtained on a Philips X'PERT MPD (PANalytical, Almelo, The Netherlands), with a CuKα between 10° and 80°. SEM was done on a FEI XL40 Sirion FEG digital scanning microscope (FEI, Hillsboro, OR, USA). TEM analysis was done using a FEI-Technai G2 Sphera microscope (FEI, Hillsboro, OR, USA) with field emission gun at 200 kV, point resolution of 0.27 nm, and a line resolution 0.14 nm.

MMD Medium Composition

Per 1 L H_2O: 0.7 g K_2HPO_4, 0.2 g KH_2PO_4, 0.66 g $(NH_4)_2SO_4$, 0.5 g sodium citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, and 3.31 g D-glucose.

Descriptions of ROS and Cellular Damage Assays

Membrane integrity was quantified by the fluorescence ratio of two dyes measured simultaneously using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes/Life Technologies, Carlsbad, CA, USA). SYTO-9 labels live (i.e. with intact membranes) bacteria with green fluorescence, and membrane – impermeant propidium iodide labels membrane – compromised bacteria with red fluorescence. The red and green fluorescence signals were measured at 620 and 530 nm, respectively, and the ratio of green:red indicated the degree of membrane integrity.

The membrane potential assay is based on the fluorescent dye 3,3'-diethyloxacarbocyanineiodide ($DiOC_2$) contained in the BacLight Bacterial Membrane Potential Kit (Molecular Probes/Life Technologies, Carlsbad, CA, USA). With a normal membrane potential, $DiOC_2$ enters the cell and shifts from a green to red fluorescence. When the membrane is depolarized, however, more $DiOC_2$ will be present extracellularly, and the ratio of red:green

fluorescence will be decreased. The red and green fluorescence signals were measured at 590 and 530 nm, respectively. A decreased red:green ratio indicates membrane depolarization.

The electron transport assay measures dehydrogenase activity with the RedoxSensor dye (Molecular Probes/Life Technologies, Carlsbad, CA, USA). Dye entering the cell is reduced by dehydrogenase and fluoresces green. The green fluorescence was measured at 530 nm. Greater green fluorescence indicates greater dehydrogenase activity.

Total ROS levels were quantified by exposing cells to 2',7'-dichlorofluorescein diacetate (DCFH-DA), and measuring the fluorescence signal of dichlorofluorescein (DCF) at 530 nm. When exposed to ROS, DCFH-DA is nonspecifically oxidized to DCF, thus the greater the fluorescence signal, the higher ROS concentration. Since the assay is nonspecific, the measured signals were converted to H_2O_2 equivalents by using H_2O_2 standards.

Superoxide converts the colorless tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-((phenylamino)carbonyl)-2H tetrazolium hydroxide, sodium salt) to formazan (a yellow/orange compound). Absorbance was measured at 490 nm to quantify superoxide levels.

Silver Speciation in MMD

Equilibrium silver speciation assuming complete dissolution of $AgNO_3$ or nano-Ag in MMD (30 ^oC, pH = 7.0) was modeled using MINEQL+ v. 4.6 software (Environmental Research Software, Hallowell, ME, USA). The chemical composition of the MMD medium entered into the software was identical for $AgNO_3$ and nano-Ag, except for the presence/absence of NO_3^- (present after $AgNO_3$ dissolution) or cysteine (present after nano-Ag dissolution). Equilibrium was evaluated for starting Ag concentrations of 2.5 mg L^{-1} (23 μM), 5.0 mg L^{-1} (46 μM), 10.0 mg L^{-1} (92 μM) and 100.0 mg L^{-1} (920 μM).

2. SUPPORTING TABLES

Table S1. List of assays, Ag concentrations and measurement times used to evaluate cellular ROS levels and oxidative damage.

Assay	Dye/Reagent	Ag Concentration (mg L^{-1})	Measurement Times (h)[a]
Membrane Integrity	SYTO-9/Propidium Iodide	0, 2.5, 10.0	0, 0.5, 1.0, 1.5, 2.0, 4.0
Membrane Potential	$DiOC_2$	0, 2.5, 10.0	0, 0.5
e^- Transport Activity	RedoxSensor	0, 2.5, 10.0	0, 0.5, 1.0
Total ROS	DCFH-DA	0, 2.5, 10.0	0, 1.0
Superoxide	XTT	0, 2.5, 10.0	0, 2.0, 4.0

[a]The elapsed time following cell culture/Ag/reagent mixing, and initial incubation. The initial incubation times were 0.25, 0.75, 0.25, 0 and 0 h for the membrane integrity, membrane potential, e^- transport activity, total ROS and superoxide assays, respectively. The time "0" measurement represented a minimal exposure time (MET) as per Horst et al. (2013), which occurred approximately after 15 minutes of establishing the reactions.

Table S2. Equilibrium silver speciation in MMD as predicted by MINEQL+ software. The percentage of the total added Ag found in each compound is shown in parentheses.

Ag (as $AgNO_3$) Concentration	Final Concentration (μM)[a] and percentage of total (in parentheses, %)			
	Ag^+	$AgNH_3^+$	$Ag(NH_3)_2^+$	$AgSO_4^-$
2.5 mg L^{-1} (23 μM)	21 (89.1%)	1.2 (5.2%)	0.3 (1.1%)	1.0 (4.5%)
5.0 mg L^{-1} (46 μM)	41 (89.1%)	2.4 (5.2%)	0.5 (1.1%)	2.1 (4.5%)
10.0 mg L^{-1} (92 μM)	83 (89.1%)	4.9 (5.2%)	1.0 (1.1%)	4.2 (4.5%)
100.0 mg L^{-1} (920 μM)	830 (89.2%)	48 (5.2%)	10 (1.1%)	42 (4.5%)

Ag (as nano-Ag) Concentration	Final Concentration (μM)[b] and percentage of total (in parentheses, %)			
	Ag^+	$AgNH_3^+$	$Ag(NH_3)_2^+$	$AgSO_4^-$
2.5 mg L^{-1} (23 μM)	21 (89.1%)	1.2 (5.2%)	0.3 (1.1%)	1.0 (4.5%)
5.0 mg L^{-1} (46 μM)	41 (89.1%)	2.4 (5.2%)	0.5 (1.1%)	2.1 (4.5%)
10.0 mg L^{-1} (92 μM)	83 (89.1%)	4.9 (5.2%)	1.0 (1.1%)	4.2 (4.5%)
100.0 mg L^{-1} (920 μM)	830 (89.2%)	48 (5.2%)	10 (1.1%)	42 (4.5%)

[a]Also predicted as present at low concentrations (<0.1%): $Ag(OH)_2^-$, AgOH and $AgNO_3$.
[b]Also predicted as present at low concentrations (<0.1%): $Ag(OH)_2^-$ and AgOH.

Table S3. Cellular total ROS (mg L^{-1} H_2O_2 equivalents) for *E. coli* and *P. aeruginosa* cultures in MMD after 60 minutes of nano-Ag or $AgNO_3$ exposure.

Treatment	*E. coli*	*P. aeruginosa*
Control	0 ± 0^a	0 ± 0^a
2.5 mg L^{-1} nano-Ag	0 ± 5.7^a	60.5 ± 2.3^b
10 mg L^{-1} nano-Ag	0 ± 28.1^a	59.8 ± 0.4^b
2.5 mg L^{-1} $AgNO_3$	323.1 ± 27.3^b	76.7 ± 3.1^c
10 mg L^{-1} $AgNO_3$	393.3 ± 34.8^b	75.4 ± 2.5^c

[a-c] Like letters in each column indicate no significant difference (*t* test, $p > 0.05$).

3. SUPPORTING FIGURES

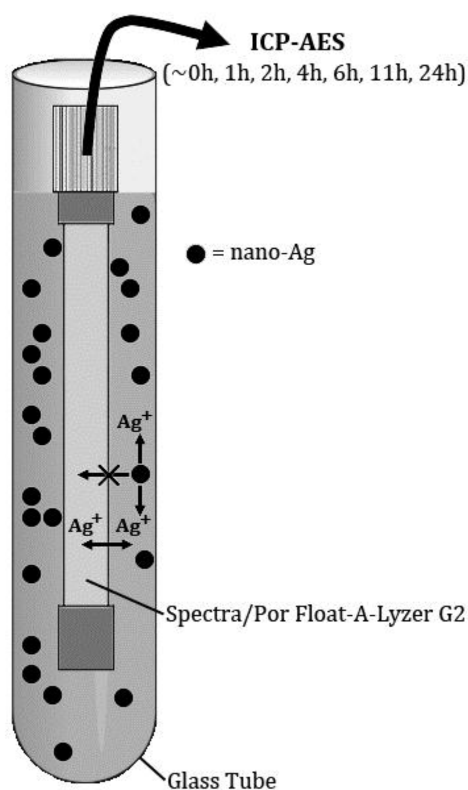

Figure S1. Schematic diagram of the apparatus used for the measurement of nano-Ag dissolution over time. A Spectra/Por Float-A-Lyzer G2 cellulose ester dialysis membrane apparatus containing the appropriate medium (H_2O, MMD or LB) was suspended in nano-Ag – amended medium. The membrane was permeable to dissolved Ag^+ ions, but not intact nano-Ag particles. Small aliquots of Ag^+ - containing medium were removed periodically from 0 – 24 h, and measured for total Ag concentration using ICP-AES. Measurements were also performed using $AgNO_3$ instead of nano-Ag.

This journal is © The Royal Society of Chemistry 2013

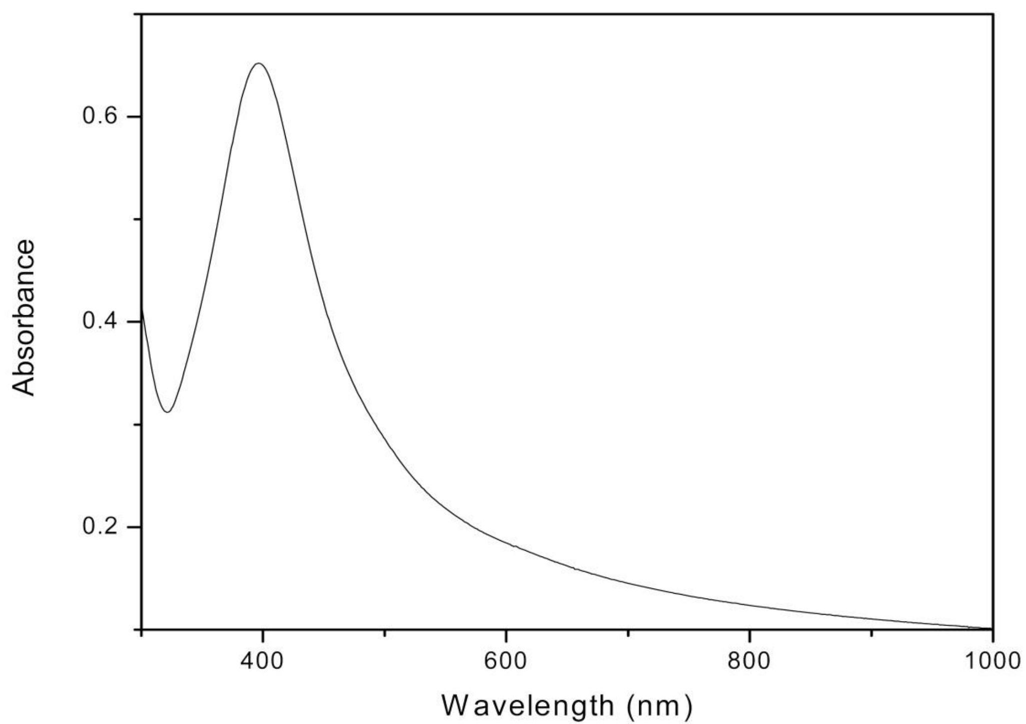

Figure S2. Optical absorption spectrum of nano-Ag. The plasmon band at approximately 400 nm is the characteristic peak for Ag.

This journal is © The Royal Society of Chemistry 2013

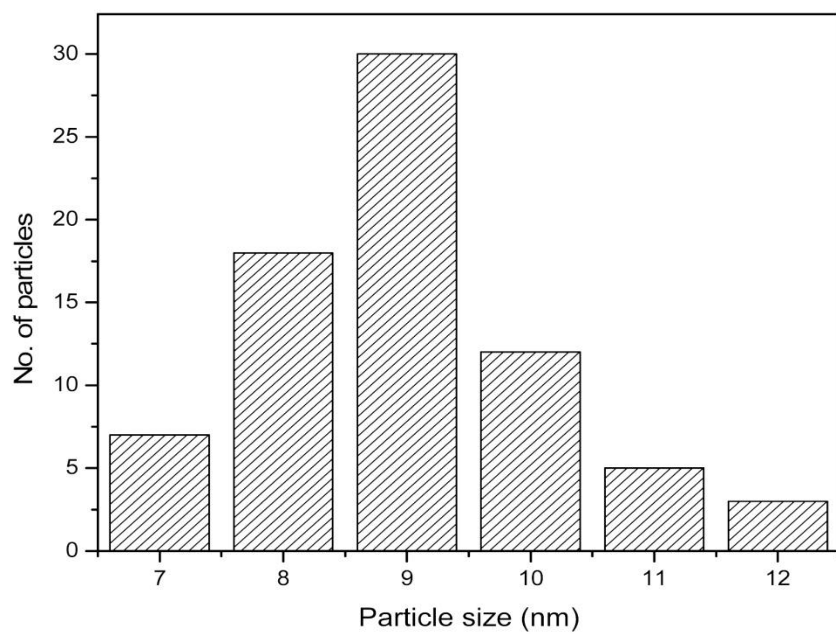

Figure S3. Particle size distribution for nano-Ag.

This journal is © The Royal Society of Chemistry 2013

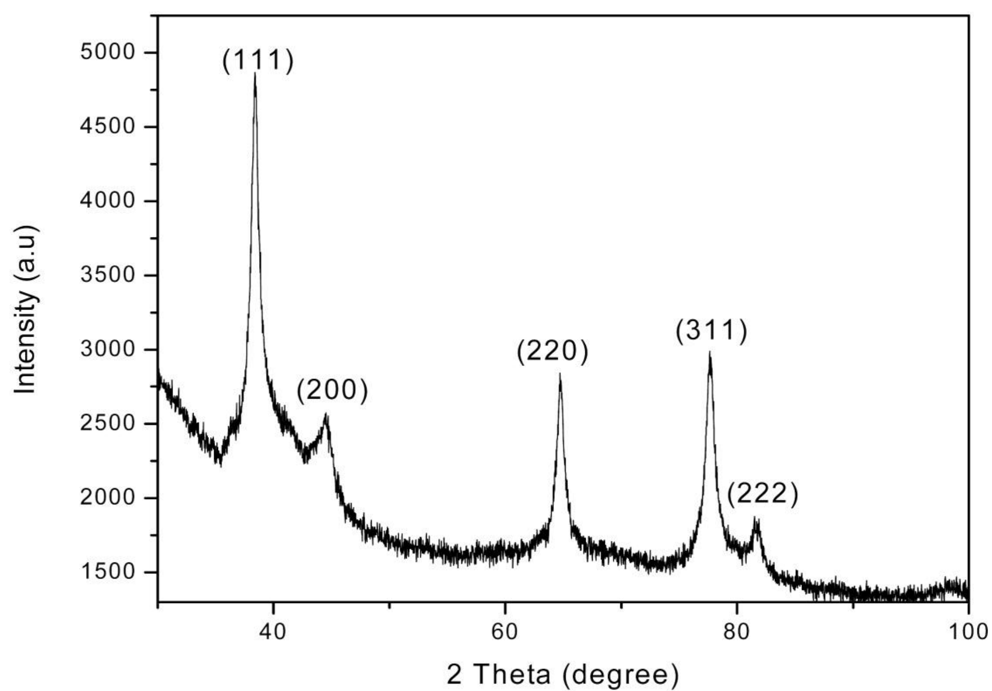

Figure S4. X-ray diffraction pattern of nano-Ag. The labeled peaks match the reported peaks for face centered cubic Ag nanoparticles with miller indices (111), (200), (220), (311), and (222).

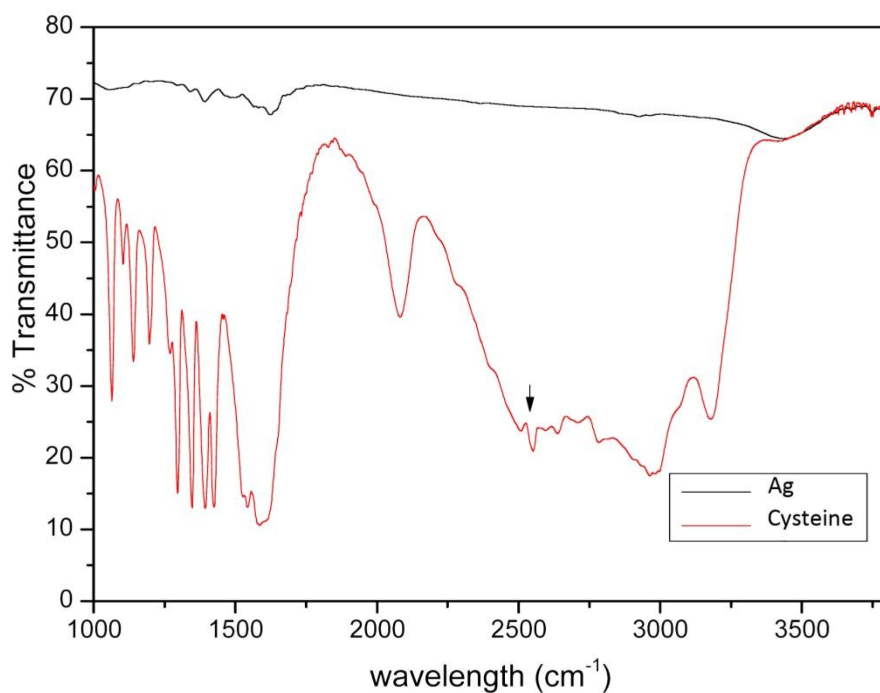

Figure S5. FTIR spectra of nano-Ag and cysteine. The spectrum of nano-Ag indicates clearly the formation of a thiol-silver bond, marked by the disappearance of a 2500 cm^{-1} peak (arrow) in free cysteine which is due to the thiol group. This peak is absent in the IR spectrum of nano-Ag, due to the formation of an S-Ag bond replacing the thiol (S-H) bond in cysteine.

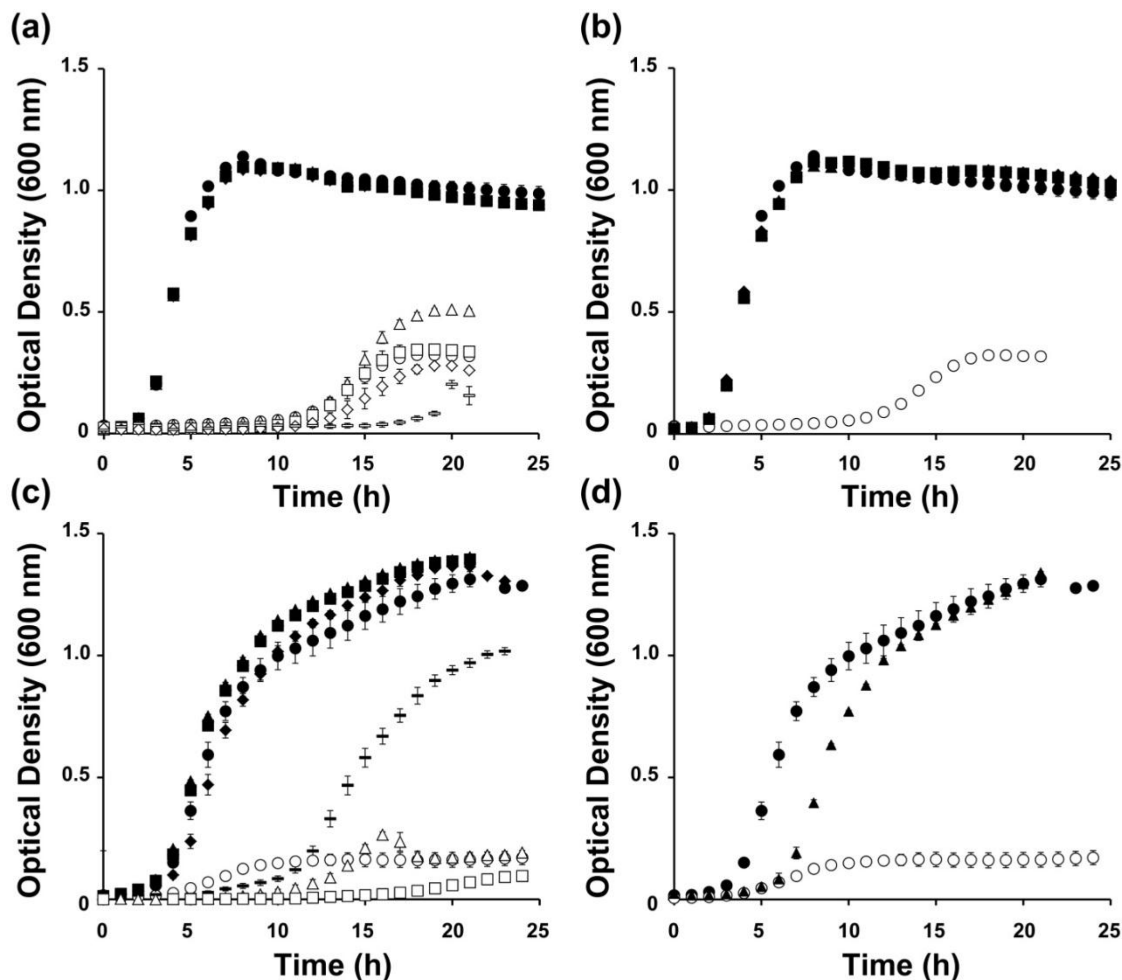

Figure S6. *E. coli* and *P. aeruginosa* growth curves in the presence of nano-Ag and $AgNO_3$. Panels a and b show *E. coli* growth with nano-Ag and $AgNO_3$, respectively. Panels c and d show *P. aeruginosa* growth with nano-Ag and $AgNO_3$, respectively. Closed symbols represent growth in LB, while open symbols represent growth in MMD. Symbol shapes indicate total Ag concentration (● = 0 mg L^{-1}, ▲ = 2.5 mg L^{-1}, ■ = 5.0 mg L^{-1}, ♦ = 10.0 mg L^{-1}, ▬ = 100.0 mg L^{-1}). Error bars represent the standard error of the mean. No data are shown where there was no growth, e.g. of either bacterial strain with any concentration of $AgNO_3$ in MMD. Also, *P. aeruginosa* did not grow with nano-Ag in MMD at either 10 or 100 mg L^{-1}. *E. coli* did not grow with $AgNO_3$ in LB at 100 mg L^{-1} and *P. aeruginosa* did not grow with $AgNO_3$ at 5, 10 or 100 mg L^{-1} in LB.

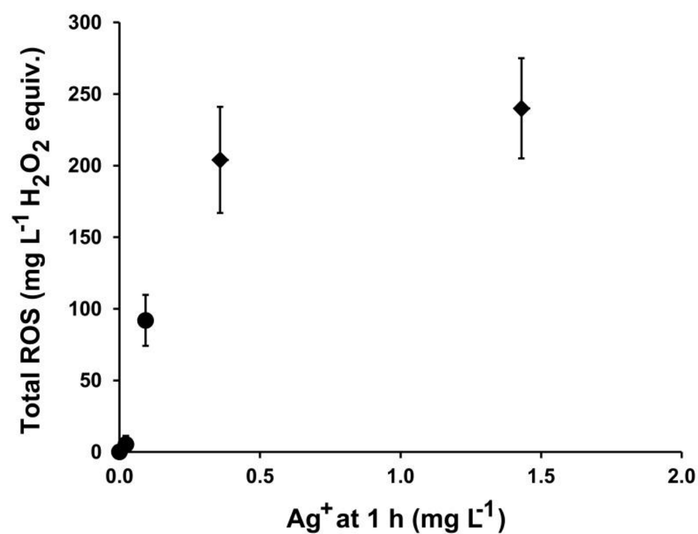

Figure S7. Total ROS versus dissolved Ag^+ ion concentration in MMD at 1 h. Circles and diamonds represent nano-Ag and $AgNO_3$ treatments, respectively. Error bars represent the standard error of the mean.

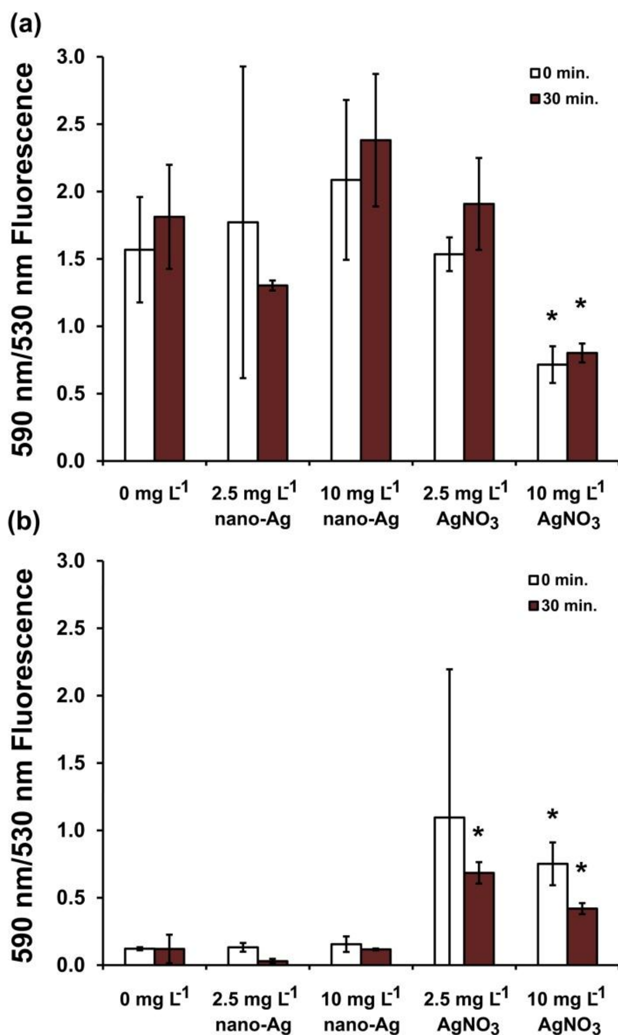

Figure S8. Fluorescence ratio (590:530 nm), indicating membrane potential, for *E. coli* (a) and *P. aeruginosa* (b) in MMD medium amended with nano-Ag or $AgNO_3$. Error bars represent the standard error of the mean. Asterisks (*) indicate values that are significantly different than the control (0 mg L^{-1}) at each time point (*t* test, $p < 0.05$).

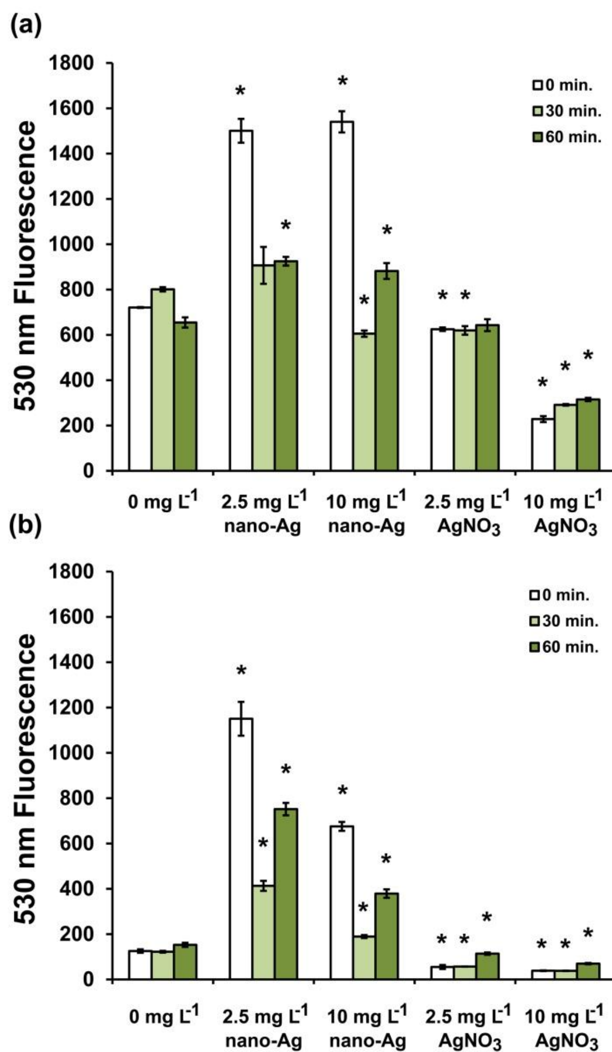

Figure S9. Fluorescence (530 nm), indicating electron transport activity, for *E. coli* (a) and *P. aeruginosa* (b) in MMD medium amended with nano-Ag or $AgNO_3$. Error bars represent the standard error of the mean. Asterisks (*) indicate values that are significantly different than the control (0 mg L^{-1}) at each time point (*t* test, $p < 0.05$).