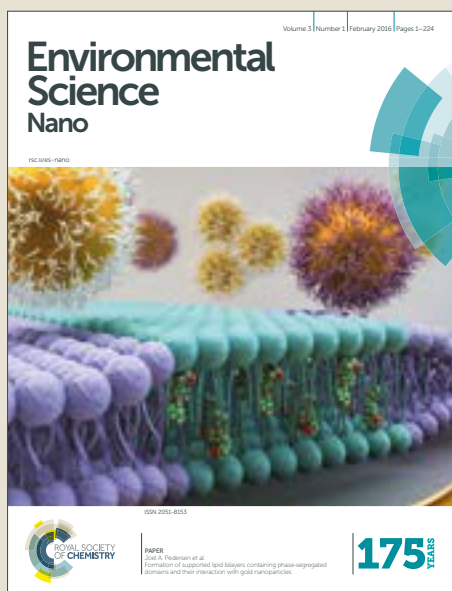


Environmental Science Nano

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Efforts to understand the environmental impact of released nanoparticles have identified some simple relationships between nanoparticle binding and toxicity to bacteria. Here, we use a panel of Gram-negative bacteria that come from diverse environmental niches to assess nanoparticle toxicity and to further understand the interaction of nanoparticles with diverse bacterial cell walls. In using such a panel, we see only a loose correlation between nanoparticle binding amounts and observed toxicity. This demonstrates that more complex biological mechanisms may be involved in nanoparticle toxicity. Using a panel comprised of environmentally-diverse bacteria can help account for biological complexity and allow identification of bacteria types that are most affected by different nanoparticles, which should improve efficiency in investigating the environmental impacts of nanoparticles.



Environmental Science: Nano

COMMUNICATION

Using an environmentally-relevant panel of Gram-negative bacteria to assess the toxicity of polyallylamine hydrochloride-wrapped gold nanoparticles

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10 Jacob,^c Catherine J. Murphy,^c Rigoberto Hernandez,^b and Christy L. Haynes^a

11 We aim to establish the effect of environmental diversity in
12 evaluating nanotoxicity to bacteria. We assessed the toxicity of 4
13 nm polyallylamine hydrochloride-wrapped gold nanoparticles to a
14 panel of bacteria from diverse environmental niches. The bacteria
15 experienced a range of toxicities as evidenced by the different
16 minimum bactericidal concentrations determined; the sensitivities
17 of the bacteria was *A. vinelandii* = *P. aeruginosa* > *S. oneidensis* MR-
18 4 > *A. baylyi* > *S. oneidensis* MR-1. Interactions between gold
19 nanoparticles and molecular components of the cell wall were
20 investigated by TEM, flow cytometry, and computational modeling.
21 Binding results showed a general trend that bacteria with smooth
22 LPS bind more PAH AuNPs than bacteria with rough LPS.
23 Computational models reveal that PAH migrates to phosphate
24 groups in the core of the LPS structure. Overall, our results
25 demonstrate that simple interactions between nanoparticles and
26 the bacterial cell wall cannot fully account for observed trends in
27 toxicity, which points to the importance of establishing more
28 comprehensive approaches for modeling environmental
29 nanotoxicity.

Introduction

31 Due to the unique physicochemical properties that arise from their
32 high surface-area-to-volume ratio, nanomaterials are increasingly
33 used in consumer products. It is inevitable that, at some stage of the
34 manufacturing, use, and disposal of such products, some of the
35 nanomaterials they contain may be released into the environment.
36 Therefore, there is a growing focus to understand the behavior of
37 engineered nanomaterials in the environment and determine their

38 potential environmental impacts.^{1,2} One can probe the potential
39 environmental impact through the use of bacterial models, which, as
40 decomposers, occupy an important trophic level; decomposers
41 recycle nutrients that can be used by primary producers.³ Therefore,
42 any effects on bacteria may impact organisms in other trophic levels,
43 making bacteria a good diagnostic for overall environmental health.
44 Often, only one bacterial model is used in nanoparticle toxicity
45 studies,^{4–6} but this can lead to results that may not be generalizable
46 across bacteria from different environments. Therefore, we have
47 assembled a panel of Gram-negative bacteria with sequenced
48 genomes that occupy different environmental niches for use in
49 nanotoxicity studies.

50 When evaluating nanoparticle toxicity to bacteria, direct
51 interactions of nanoparticles to the bacterial surface play a role in
52 the toxicity, with several studies demonstrating a correlation
53 between amount of NPs bound to bacteria and observed toxicities.^{7–}
54 10 It has been shown that bound NPs can rupture the bacterial cell
55 membrane,^{11,12} lead to alterations in the membrane potential,¹³
56 release ions that are localized right at the bacterial surface,¹⁴ and
57 generate reactive oxygen species at the cell membrane.¹⁵ In previous
58 work, we have shown that a main component of the Gram-negative
59 bacterial surface, lipopolysaccharides (LPS), are important in
60 facilitating the binding of nanoparticles with the surface of the model
61 bacterium, *S. oneidensis* MR-1.¹⁶ There are two broad classes of LPS,
62 designated as either rough or smooth. Rough LPS have a lipid A
63 region that anchors the LPS into the membrane and an
64 oligosaccharide portion that is bound to the lipid A. By contrast,
65 smooth LPS have both lipid A and core oligosaccharide regions, with
66 the addition of an O-antigen, a polysaccharide domain bound to the
67 core oligosaccharide, elongating the overall LPS structure. Based on
68 the clear role of LPS in binding nanoparticles, it makes sense to
69 generate a panel that focuses on variation in LPS structure. Such a
70 panel would exclude Gram-positive bacteria, which are also
71 important environmental organisms, but allows us to focus on
72 specific surface chemistry differences between the bacteria used.

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Electronic Supplementary Information (ESI) available: Known LPS structures from the bacterial panel, citrate-capped AuNP synthesis, quantification of free PAH, TEM of PAH AuNP binding to bacteria, flow cytometry gating method and supplemental data, and simulation of distance between LPS phosphorus and PAH center of mass. See DOI: 10.1039/x0xx00000x

Table 1. The bacteria in the panel come from a range of habitats and have different respiration abilities. The panel includes bacteria with either rough or smooth LPS presented on their surface. Each bacterium in the panel has an important role in the environment.

Bacterial Strain	Habitat	Respiration	LPS type	Environmental Role
<i>Azotobacter vinelandii</i> UW	Soil ⁴⁸	Obligate aerobe ⁴⁸	Smooth ⁴⁹	Nitrogen cycle
<i>Acinetobacter baylyi</i> ADP1	Soil, sediment, aquatic ¹⁸	Obligate aerobe ¹⁸	Smooth ¹⁸	Metabolize aromatic compounds
<i>Shewanella oneidensis</i> MR-1	Soil, marine ²⁰	Facultative anaerobe ²⁰	Rough ²⁵	Geochemical nutrient cycle
<i>Shewanella oneidensis</i> MR-4	Soil, marine ²⁰	Facultative anaerobe ²⁰	Rough ²¹	Geochemical nutrient cycle
<i>Pseudomonas aeruginosa</i> PAO1	Ubiquitous ⁵⁰	Obligate aerobe ⁵⁰	Smooth ⁵¹	Metabolic diversity

The five bacteria that make up the bacterial panel introduced in this manuscript include *Azotobacter vinelandii* UW, *Acinetobacter baylyi* ADP1, *Shewanella oneidensis* MR-1, *Shewanella oneidensis* MR-4, and *Pseudomonas aeruginosa* PAO1. They are a mix of Gram-negative bacteria that have smooth or rough LPS on their surface. Differences between the bacteria in the panel are highlighted in Table 1. In the environment, *A. vinelandii* has an important role in the nitrogen cycle since it is capable of fixing nitrogen even in the presence of atmospheric oxygen.¹⁷ The smooth LPS of *A. baylyi* likely has branched O-antigens since that is characteristic of the genus *Acinetobacter*;¹⁸ this bacterium is capable of great metabolic diversity, notably in its ability to metabolize aromatic compounds that are often products of plant degradation.¹⁹ *S. oneidensis* MR-1 has an important environmental role in geochemical nutrient cycling since it is capable of reducing a wide variety of metals.²⁰ Similarly, *S. oneidensis* MR-4 is also capable of dissimilatory reduction of many different metals.²¹ Finally, *P. aeruginosa* PAO1 is an obligate aerobe that can adapt to live in many different environments due to its metabolic diversity. *P. aeruginosa* is often used as a biofilm formation model; biofilms may serve as a sink for NPs entering the environment, making it likely that *P. aeruginosa* would encounter nanomaterials that are released into the environment.²²

Since these bacteria occupy diverse environmental niches and have different surface compositions, we expect that they will be representative of bacteria in the environments that nanoparticles may end up in. These differences should also make them suitable for showing a range of responses to nanoparticle exposure so that a particular nanoparticle is not deemed non-toxic because a single bacterial species happens to be tolerant to it. The motivation for noting the different LPS structures on each bacterial species' cell surface is that the differences in LPS length and composition may impact their interaction with the NPs used in this study. The saccharide portions of the LPS structures of *P. aeruginosa*,^{23,24} *S. oneidensis* MR-1,²⁵ and *S. oneidensis* MR-4²¹ have been elucidated (Fig. S1), but the LPS structures of the other bacteria in the panel are not as well characterized. The charges of these LPS structures as well as other LPS structures used in simulations in this manuscript are given (Table 2).

To demonstrate the use of the bacterial panel in this manuscript, each species is exposed to 4-nm-diameter polyallylamine hydrochloride (PAH)-coated gold nanoparticles (AuNPs). AuNPs were used in this study due to their chemical inertness, size/shape tunability, and ease of characterization.^{26,27} Polyelectrolyte coating is an industrially-relevant modification of materials as these functionalized materials have many applications in diverse fields,^{28–30} and this particular coating is known to interact with bacterial surfaces and cause membrane disruption as its toxicity mechanism.^{7,31} While there is limited work studying PAH AuNPs with the bacteria in this panel,³² in previous work, the toxicity of PAH AuNPs was investigated for *S. oneidensis* MR-1 using a colony counting method.⁷ In this manuscript, the effects of the PAH AuNPs to each bacterium are noted by determining the minimum bactericidal concentration (MBC). The MBC is defined as the lowest nanoparticle concentration that kills at least 99% of the bacteria. This is a facile method to demonstrate the different responses that each bacterium has to NP exposure. To test the hypothesis that extent of NP binding to the bacterial cell surface correlates with toxicity, transmission electron microscopy (TEM) is used to visualize and flow cytometry is used to quantify PAH AuNP binding to each species. In parallel, a molecular dynamics simulation was used to calculate

Table 2. The number of phosphates and charge of the LPS core polysaccharide structure are provided for several bacterial strain variants (first three rows) and model structures (remaining four rows). O-antigens were entirely absent in all rough model structures and only two O-antigens are included in the "smoother" structure. The PAH used in the simulations has a charge of +10 leading to the total charges listed in the final column. Negative and positive charges were neutralized in the simulations using the corresponding number of sodium cations or chloride anions, respectively.

Bacterial Strain	Number of Phosphate Units	Charge of LPS	Charge of LPS + PAH
<i>S. oneidensis</i> MR-1	5	-7	+3
<i>S. oneidensis</i> MR-4 variant 1	4	-6	+4
<i>S. oneidensis</i> MR-4 variant 2	6	-5	+5
Rough <i>P. aeruginosa</i> PAO1 variant	6	-12	-2
"Smoother" <i>P. aeruginosa</i> PAO1 variant	6	-14	-4
Rough <i>S. typhimurium</i>	4	-10	0
Rough <i>E. coli</i>	5	-9	+1

1 relative association energies of the PAH that caps the nanoparticles
2 for different LPS structures to determine if the presentation of
3 negatively charged phosphate groups facilitates the interaction of
4 LPS with the amine groups of PAH. Taken together, the results
5 demonstrate that a simple hypothesis related to the molecular
6 character of the LPS is not sufficient to explain the nanoparticle
7 association and toxicity results. This is a benefit to using such a panel,
8 as it identifies instances where the biological complexity can mask
9 simple, expected trends. Indeed, this panel does reveal which
10 bacterial strains are most critical for follow-on work and facilitates
11 the formulation of further hypotheses. While AuNPs are the focus of
12 this work, this panel can be adapted for use with a range of
13 nanomaterials.

14 Materials and Methods

15 Materials

16 Magnesium sulfate (MgSO_4), sucrose, sodium molybdate dihydrate
17 ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic
18 acid (HEPES), gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), sodium
19 citrate tribasic dihydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$), polyallylamine
20 hydrochloride (PAH, MW 17.5 kDa), sodium borohydride (NaBH_4),
21 and sodium chloride (NaCl) were purchased from Sigma-Aldrich
22 (Milwaukee, WI). Potassium phosphate dibasic trihydrate
23 ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) was purchased from Mallinckrodt (Phillipsburg, NJ).
24 Potassium phosphate monobasic (KH_2PO_4) was obtained from J.T.
25 Baker (Center Valley, PA). Dulbecco's phosphate-buffered saline was
26 purchased from Corning (Aurora, CO), LB broth and agar were
27 obtained from BD Difco (Franklin Lakes, NJ). SYTO9 nucleic acid stain
28 was obtained from Molecular Probes (Waltham, MA). Calcium
29 chloride (CaCl_2) and ferrous sulfate (FeSO_4) were purchased from
30 Fisher Scientific (Rockford, IL). Absolute anhydrous 99.5% ethanol
31 was obtained from Pharmco-Aaper (Brookfield, CT). All chemicals
32 were used as received. Deionized water (18.2 M Ω) was purified using
33 a Milli-Q Millipore water purification system (Billerica, MA).
34 *Shewanella oneidensis* MR-1 was obtained from Jeffrey Gralnick
35 (Dept of Microbiology, University of Minnesota). *Shewanella*
36 *oneidensis* MR-4 was obtained from Daad Saffarini (Dept of Biological
37 Sciences, University of Wisconsin – Milwaukee). *Acinetobacter baylyi*
38 (ATCC® 33305™), *Azotobacter vinelandii* (ATCC® 13705™), and
39 *Pseudomonas aeruginosa* (ATCC® 47085™) were purchased from the
40 American Type Culture Collection (Manassas, VA).

41 PAH-coated AuNPs (PAH AuNPs)

42 PAH AuNPs were synthesized by polyelectrolyte wrapping of as-
43 synthesized citrate-capped AuNPs (see ESI for synthesis of citrate-
44 capped AuNPs).³³ To the approximately 3.2 L of as-synthesized
45 citrate-capped AuNPs, 32.0 mL of 100 mM NaCl and 100.0 mL of a
46 PAH solution (MW 17.5 kDa) (10 mg/mL in 1 mM NaCl) was added
47 with vigorous stirring. The solution was stirred overnight and
48 concentrated to around 30 mL by diafiltration cassettes (Tangential
49 Flow Filtration Capsules, 50K MWCO, VWR). The concentrated PAH
50 AuNPs were purified by centrifugation at 13,000 $\times g$ for 55 min.

51 AuNP characterization

52 PAH AuNPs were characterized post-synthesis by UV-vis extinction
53 spectroscopy, TEM, and ζ -potential measurement. The size and ζ -
54 potential for the PAH AuNPs were also characterized in the exposure
55 medium using UV-vis extinction spectroscopy and ζ -potential
56 measurement. UV-vis extinction spectra post-synthesis were
57 obtained on a Cary 500 UV-vis-NIR spectrophotometer and the
58 measurements taken in exposure medium were obtained on an
59 Ocean Optics USB2000 spectrophotometer. For TEM studies, 5 μL of
60 a dilute solution of AuNPs was drop-cast onto a TEM grid (Ted Pella,
61 Redding, CA), and the AuNP sample images were taken with a JEOL
62 2100 TEM. ζ -potential measurements were obtained using a
63 Brookhaven ZetaPALS instrument.

64 Bacterial Culture Conditions

65 Bacteria were stored at -80 °C until ready for use. For *S. oneidensis*
66 MR-1, *S. oneidensis* MR-4, *A. baylyi*, and *P. aeruginosa*, the
67 appropriate bacterial stock was plated on a sterilized Luria-Bertani
68 (LB) agar plate and incubated at 30 °C. Two colonies were inoculated
69 in 10 mL of LB broth and incubated overnight. For *A. vinelandii*, plates
70 with Burk's medium adapted from Newton, et al³⁴ were used and
71 two colonies were inoculated in 10 mL of Burk's medium. The
72 bacteria at late log phase were centrifuged at 750 $\times g$ for 10 min and
73 washed with Dulbecco's phosphate-buffered saline (DPBS) before
74 resuspension in HEPES buffer (2 mM HEPES, 25 mM NaCl, pH=7.4) to
75 the appropriate cell density.

76 Minimum Bactericidal Concentration Determination

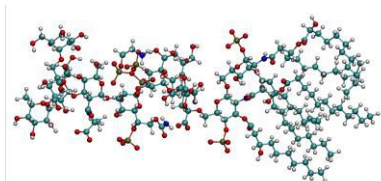
77 The cells were diluted in HEPES buffer to a cell density of 2×10^6
78 cells/mL. Cells were either exposed to PAH-AuNPs (2.81, 0.28, or
79 0.028 ppm) or to free PAH (21.16, 2.116, or 0.2116 ppm) for 10
80 minutes by mixing 180 μL of bacterial suspension with 20 μL of PAH
81 AuNP or free PAH suspension. Free PAH controls were performed
82 since the PAH AuNP suspension contained free PAH that was left over
83 from the wrapping process. The concentrations of free PAH present
84 in the AuNP suspensions were determined using a fluorescamine
85 assay as previously described (see ESI for fluorescamine assay
86 experimental details)³⁵ and were used to distinguish the impact of
87 free polyelectrolyte from polyelectrolyte presented on the AuNP
88 surface. Following the 10 minute exposure, six 10 μL drops of each
89 treatment were dropped onto a dried, UV-sterilized LB agar plate
90 (Burk's medium plates were used for *A. vinelandii*). Once the drops
91 absorbed into the agar, the plates were incubated upside-down in a
92 30 °C incubator overnight. The PAH AuNP and free PAH
93 concentration that killed at least 99% of the bacteria were recorded.

95 Transmission Electron Microscopy Analysis

96 Before taking images of nanoparticle-exposed bacteria with the TEM,
97 the samples had to be embedded in epoxy resin.^{7,36} At an optical
98 density of 0.8 in HEPES, the bacteria were exposed to 0.281 ppm PAH
99 AuNPs for 10 min and then washed three times in 0.1 M cacodylate
100 buffer. The cells were fixed using 2.5% glutaraldehyde in 0.1 M
101 cacodylate buffer. This step proceeds for 50 min, flipping the pellet
102 after 25 min to ensure fixation. The pellet is then washed (without
103 resuspension) three times in 0.1 M cacodylate buffer.

To dehydrate the cells, ethanol was used at increasing concentrations in water (30%, 50%, 70%, 80%, 95%, and 100% ethanol). They were rinsed three times with propylene oxide before being incubated with a 2:1 propylene oxide:resin mix for 2 hours uncovered. Then they were incubated with 1:1 propylene oxide:resin overnight followed by 1:1 propylene oxide:resin for 4 hours and pure resin overnight. After replacement with fresh resin, the samples were incubated at 40 °C for 24 hours and then 60 °C for 48 hours. The samples were cut into ~70-nm-thick sections using a LEICA EM UC6 ultramicrotome and stained with uranyl acetate and lead citrate to improve image contrast. The sections were placed on 200 mesh copper grids that have Formvar and carbon supports, and images were taken using a Tecnai T12 transmission electron microscope using an operating voltage of 120 kV.

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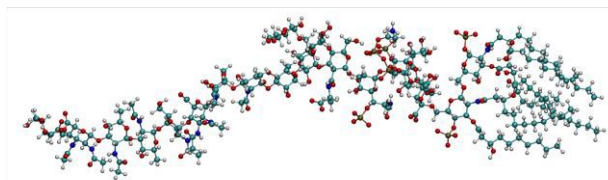


Figure 1. Ball-and-stick renderings of representative structures of a) rough LPS and b) rough LPS with two added O-antigen units onto the *P. aeruginosa* LPS structure.

Flow Cytometry Analysis

After the bacteria reached the late log phase, they were washed in DPBS and resuspended in HEPES buffer. Bacterial suspensions at 4×10^8 cells/mL were exposed to 2.81 ppm PAH AuNPs for 10 min and then incubated with 3.34 mM SYTO9 dye at room temperature for 15 min. The samples were analysed with a Becton Dickinson LSRII SORP flow cytometer with a 20 mW, 488 nm laser, using a control set of bacteria not exposed to NPs to draw the gates. SYTO9 fluorescence was used to distinguish bacteria from other debris in the sample, and light scattering was used to determine which bacteria had associated AuNPs. In total, each sample was done in triplicate, collecting 20,000 events in each run.

Computational Models and Simulation

The use of molecular dynamics simulations to characterize the large-scale association and relaxation of LPS with the PAH presented on the AuNP surface can yield insight into the underlying chemistry by resolving which sites, or sets of sites, induce interactions.^{37,38} Representative model structures of the LPS molecules found on the surface of *P. aeruginosa*, *Salmonella typhimurium*, and *Escherichia coli* have been prepared. While these three structures are not perfect matches for the bacteria in this panel (largely because not all the LPS structures are well-known), they represent a range of LPS structures and should still yield insight about critical interaction features, since

they exhibit different overall charges and have differing numbers of phosphate units in their structure. Several force fields have been developed for prediction of interfacial properties of biological materials and their interactions with inorganic and organic nanostructures.^{39–42} We employed the CHARMM36 force field because it is a transferable potential that has been widely used and benchmarked, and it presented no challenges to the numerical convergence in the current studies. These structures have been energy minimized and equilibrated in the presence of 13,000 – 25,000 explicit (TIP3P) water molecules (depending on the size of the system) through equations of motion driven by the CHARMM36 force field. The mixture of PAH, LPS molecules, and water is neutralized through the addition of counter-ions that corresponds to the number of sodium cations or chloride anions needed to neutralize the “Charge of LPS + PAH” column of Table 2. For simplicity, we use a 10-mer PAH construct as it provides a balance between the non-chain like monomer and computationally expensive long-chain polymers with hundreds or more monomeric units. The chemical and molecular structure of the selected LPS have been obtained from known properties of the LPS from the chosen bacteria listed above. Each trajectory was then propagated for 19–20 nanoseconds at a cost of 65–75 hours of computer time on the XSEDE Bridges regular memory nodes with 2.3 GHz Intel Xeon EP-Series CPUs and 128 GB memory per CPU. In all cases, the PAH approaches the LPS molecule, allowing us to consider the time to approach and the location of the approach as figures of merit (or observables).

A computational study was performed to investigate PAH binding using a rough LPS model and a LPS construct with “smoother” character. The LPS models used here vary according to the corresponding incorporation of O-antigens. The addition of two O-antigen units to the rough *P. aeruginosa* LPS was constructed using the CHARM-GUI⁴³ and models a smoother construct (Fig. 1) useful for our systematic study. The interaction between PAH and the LPS of

Table 3. Minimum bactericidal concentration values observed for each bacterium after exposure to PAH AuNPs and to free PAH. The values in parentheses after the MBC for PAH AuNPs indicates the amount of free PAH present in that concentration of PAH AuNPs as determined by the fluorescamine assay.³⁵ (Ex: 0.281 ppm of PAH AuNPs contains a free PAH concentration of 2.12 ppm). The MBCs were determined in at least triplicate measurements.

Bacteria	MBC _{PAH AuNPs} (ppm)	Free PAH present at PAH AuNP MBC concentration (ppm)	MBC _{Free PAH} (ppm)
<i>A. vinelandii</i> UW	≤0.0281	≤0.212	2.12
<i>P. aeruginosa</i> PAO1	≤0.0281	≤0.212	>21.2
<i>S. oneidensis</i> MR-4	0.281	2.12	2.12
<i>A. baylyi</i> ADP1	2.09	15.8	14.8
<i>S. oneidensis</i> MR-1	≥2.81	≥21.2	≥21.2

1 *P. aeruginosa*, *E. coli*, and *S. typhimurium* (structures shown in Fig.
2 S5), was observed through similar molecular dynamics trajectories.

3 The strength of the association of PAH to each LPS was evaluated
4 from trajectory simulations to better understand specific
5 interactions between the polyelectrolytes and LPS with different
6 structures. Specifically, the changes in the interaction energies
7 between the LPS and PAH are calculated after the complete
8 simulation trajectories are obtained. The effects of the water
9 molecules and ions on these energies are subtracted. Consequently,
10 the reported energies include only the contributions from the
11 interaction between the polyelectrolytes and LPS molecules.

12 Results and Discussion

13 Synthesis and Characterization of AuNPs

14 We first verified the size of the citrate-capped AuNPs before and
15 after functionalization with PAH using several methods. Citrate-
16 capped AuNPs in solution were validated after synthesis by UV-Vis
17 spectroscopy⁴⁴ (Fig. S2), which demonstrated that nanoparticle
18 diameter was approx. 4 nm. After functionalization with PAH, TEM
19 analysis indicated that the PAH AuNPs possessed a core diameter of
20 4.2 ± 1.2 nm ($n > 200$), and a representative TEM image is shown (Fig.
21 2). The ζ -potential of the PAH AuNPs was 46.59 ± 2.63 mV. Taken
22 together, these results demonstrate that positively charged PAH
23 AuNPs were synthesized with uniform size distribution. In the
24 exposure medium, the size of the PAH AuNPs were determined to be
25 12 ± 2 nm by UV-vis extinction spectroscopy, indicating there was
26 some affiliation of the nanoparticles to each other during the
27 exposure. The ζ -potential of the PAH AuNPs was 34 ± 2 mV, which
28 shows a slight reduction in ζ -potential in HEPES buffer, but the
29 magnitude is large enough for the particles to remain stable in
30 suspension.

31 Minimum Bactericidal Concentration (MBC) Determination

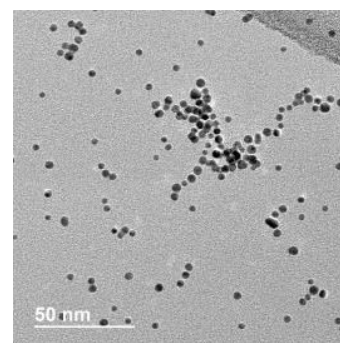


Figure 2. A representative TEM image of PAH AuNPs. Core diameter was determined to be 4.2 ± 1.2 nm ($n > 200$).

COMMUNICATION

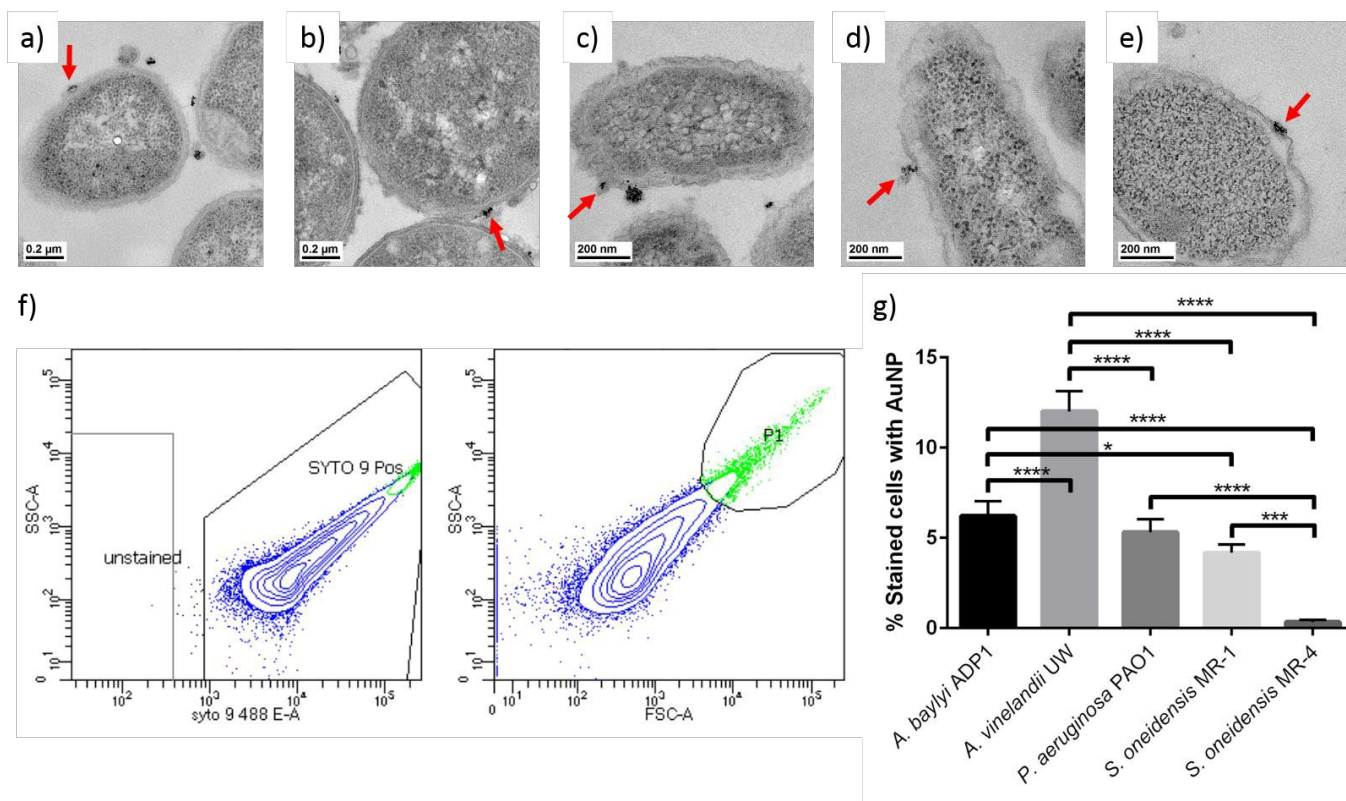
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To test their toxicity, the MBCs were determined for PAH AuNPs and free PAH (Table 3). Each of the bacteria had different sensitivities to the toxicants used in this study. The sensitivities of each bacterium to PAH AuNPs are *A. vinelandii* = *P. aeruginosa* > *S. oneidensis* MR-4 > *A. baylyi* > *S. oneidensis* MR-1, which was tolerant to all concentrations of PAH AuNPs used in this study. The trend identified by MBC shows that the toxicity cannot be as easily predicted simply based on the smooth or rough character of bacterial LPS as originally hypothesized, since the observed sensitivities do not follow a pattern correlated with LPS type. For three of the bacteria (*A. baylyi*, *S. oneidensis* MR-1, *S. oneidensis* MR-4), the toxicity of the PAH AuNPs is explained by the free PAH that is present in those suspensions, which is consistent with previous findings.³⁵ For *A. vinelandii* and *P. aeruginosa*, a nanospecific effect is seen for the PAH AuNPs since the concentration of free PAH required to kill 99% of these bacteria was higher than the concentration present in the toxic PAH AuNP suspensions. The wide range of sensitivities to both PAH AuNPs and PAH demonstrates the importance of using a bacterial panel when assessing nanoparticle properties that impact toxicity. From this experiment, the two bacteria exhibiting a nanoparticle effect have been identified to be used for follow-on work to study the

Figure 3. Transmission electron micrographs showing association of PAH AuNPs with a) *A. baylyi* ADP1, b) *A. vinelandii* UW, c) *P. aeruginosa* PAO1, d) *S. oneidensis* MR-1, and e) *S. oneidensis* MR-4. The red arrows show an example of PAH AuNP attachment to the bacterial cell wall. Representative flow cytometry data for f) *A. baylyi* exposed to 2.81 ppm PAH AuNPs. The left plot was used to identify cells based on the presence of SYTO9 stain, which is the boxed region of events labeled "SYTO 9 Pos". The right plot contains only the cells present in the boxed region of the left plot, and the events with both high side scattering and forward scattering were the population of cells with bound AuNPs. This gate was drawn using the maximum scattering seen in cells that were not exposed to PAH AuNPs. The blue dots correspond to cells stained with SYTO9 and the green events are stained bacterial cells that are bound to AuNPs. From flow cytometry, the percentage of cells that were bound to AuNPs are shown for the bacterial species after exposure to g) 2.81 ppm PAH AuNPs. * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$.



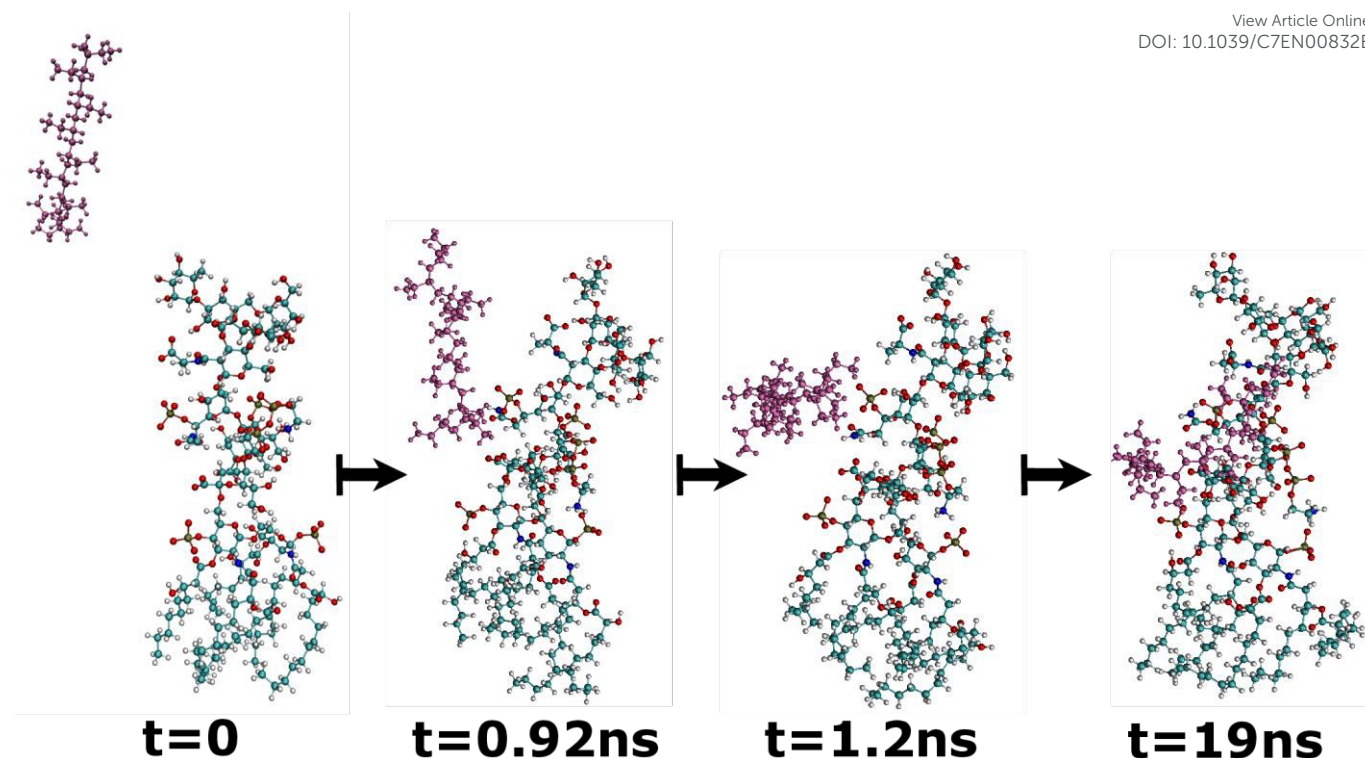


Figure 4. Snapshots of LPS from *P. aeruginosa* (in color-coded atoms) and PAH (with atoms in magenta) during a 19 ns simulation of the two molecules in explicit water (not shown). The PAH can be seen migrating toward the phosphate groups of the LPS.

1 mechanism of toxicity for this nanoparticle type, whereas the other
2 three bacteria were merely experiencing the toxicity of free PAH.

3 Nanoparticle Association with Bacteria

4 Post-exposure to PAH AuNPs, the binding was visualized using TEM,
5 with examples where binding is visible shown in Fig. 3a-e. Dark field
6 TEM was utilized to confirm the presence of the diffracting AuNPs as
7 described previously (see ESI for dark field TEM images).⁴⁵ The
8 images show that bound PAH AuNPs cover just a small area of the
9 bacterial cell envelope, with upwards of tens of nanoparticles bound
10 to any particular bacterium. The binding was quantified using flow
11 cytometry (Fig. 3f-g). Based on flow cytometry data, at 2.81 ppm PAH
12 AuNP, binding was seen for all bacteria except *S. oneidensis* MR-4,
13 which showed minimal binding. The order of binding from greatest
14 to least is *A. vinelandii* ($12 \pm 1\%$), *A. baylyi* ($6.2 \pm 0.8\%$), *P. aeruginosa*
15 ($5.3 \pm 0.7\%$), *S. oneidensis* MR-1 ($4.2 \pm 0.5\%$), and *S. oneidensis* MR-4
16 ($0.3 \pm 0.1\%$). These data show that, in general, the bacteria with
17 smooth LPS exhibit higher AuNP binding than those with rough LPS,
18 although for *P. aeruginosa* and *S. oneidensis* MR-1, these binding
19 amounts are very similar. We speculate that this is because the O-
20 antigen of smooth LPS generally has a larger number of negatively-
21 charged sites for cationic nanoparticles to interact with than rough
22 LPS.⁴⁶

23 Comparing the binding data with toxicity data reveals some
24 interesting observations. Namely, there are instances where there
25 are similar binding amounts but different observed toxicities (i.e. *P.*
26 *aeruginosa* and *S. oneidensis* MR-1) and where there is similar
27 toxicity but a different amount of binding (*A. vinelandii* and *P.*
28 *aeruginosa*). It is often assumed that direct nanoparticle interactions

29 with the cell envelope drive toxicity, and these simple relationships
30 between molecular components of the cell wall and nanoparticle
31 properties can and have been identified.⁷ Once you start
32 incorporating a wider range of organisms, some of these simple
33 relationships start to be masked by an increasing biological
34 complexity. This indicates that there are more complex mechanisms
35 involved in this interaction, and identifying these other mechanisms
36 will be important for each nanoparticle/bacterial interaction and can
37 lead to insight into that biological complexity.

38 Computational model results

39 While the experiments performed cannot be done at the same time-
40 scale as the computational models, simulations are used here to
41 derive some molecular-level insight about the interaction between
42 the PAH on the nanoparticle surface (modeled as a 10-mer) and LPS.
43 Representative snapshots of the motion of PAH toward LPS (from *P.*
44 *aeruginosa*) are shown in Fig. 4. In the first simulation, the
45 electrostatic association energies of PAH with rough LPS extracted
46 from *P. aeruginosa*, *S. typhimurium*, and *E. coli* are monitored. The
47 configuration of these rough LPS are shown in Fig. S5. The number of
48 phosphates in the core region of the LPS from *S. typhimurium* is one
49 unit less than that of *E. coli* and two units less than the *P. aeruginosa*
50 LPS structure, allowing consideration of the impact of the core
51 phosphate on association with PAH. Overall charge differences

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1 between the structures also allows for consideration of the impact of
2 charge.

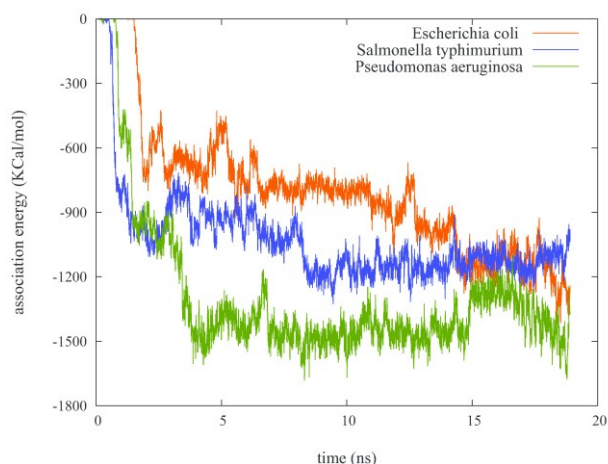


Figure 5. Electrostatic energies of the LPS/PAH association during a 19 ns simulation of 10-mer PAH interacting with the rough LPS of *P. aeruginosa* (green), *E. coli* (red), and *S. typhimurium* (blue) shown in Fig. S5.

3 The electrostatic association energies are shown in Fig. 5. The
4 PAH molecule was seen to move towards phosphate units of the core
5 region in the trajectories; the distances between the phosphorus
6 atoms and PAH through the 19 ns simulation are shown in the ESI
7 (Figs. S6-S8). These graphs suggest that the total charge of the LPS
8 and the number of the phosphate units are important parameters in
9 determining PAH association. The *E. coli* LPS has one unit less total
10 negative charge compared to that of *S. typhimurium* (Table 2).
11 Consequently, we observe a slower association of PAH to *E. coli* LPS.
12 However, the *E. coli* LPS has one phosphate unit more than the LPS
13 of *S. typhimurium*. The combination of these two competing factors
14 is one possible reason for the observation of nearly equal association
15 energy values at the end of the simulations for *E. coli* and *S.*
16 *typhimurium* LPS. On the other hand, the LPS of *P. aeruginosa* has a
17 higher negative charge and more phosphate units, leading to a
18 quicker association of PAH to LPS that is also stronger at the end of
19 the simulation than the other two bacteria.

20 We also investigated the different electrostatic association
21 energies of PAH with increasingly smooth LPS character. The degree
22 of PAH association to the rough LPS-exhibiting *P. aeruginosa* was
23 compared to that of a smoother construct of *P. aeruginosa* with two
24 added O-antigen units and is shown in Fig. 6. The LPS structure of *P.*
25 *aeruginosa* with two added O-antigen units has a total charge of -14.
26 The changes in the LPS/PAH electrostatic association energies and
27 the distance between a selected phosphorus atom and the PAH
28 center of mass are shown in Fig. 6. The traces in Fig. 6 suggest that
29 PAH associates to the O-antigen sections of the smoother LPS
30 molecule at early stages of the simulation and finally moves toward
31 the phosphate units in the core region. Moreover, the addition of
32 two O-antigen units does not significantly slow down the overall
33 movement of PAH towards the core region of LPS. Typically, smooth
34 LPS has many more than the two repeats of O-antigen monomer
35 used here. In the case of the B-band in *P. aeruginosa*, for example,
36 there can be greater than 50 repeats. Future computational work will
37 build toward this more complex LPS structure. Since the B-band LPS

38 structure of *P. aeruginosa* PAO1 has many more negatively charged
39 sites—because many of its sugars are amino-derivatized uronic acid
40 or fucose moieties,⁴⁶—this band is expected to offer more binding
41 sites for cationic particles than the more hydrophobic A-band.
42 Therefore, smooth LPS should have many more binding sites that
43 extend further into solution than those of rough LPS. This is likely why
44 we see more binding to the bacteria with smooth LPS in our
45 experimental work.

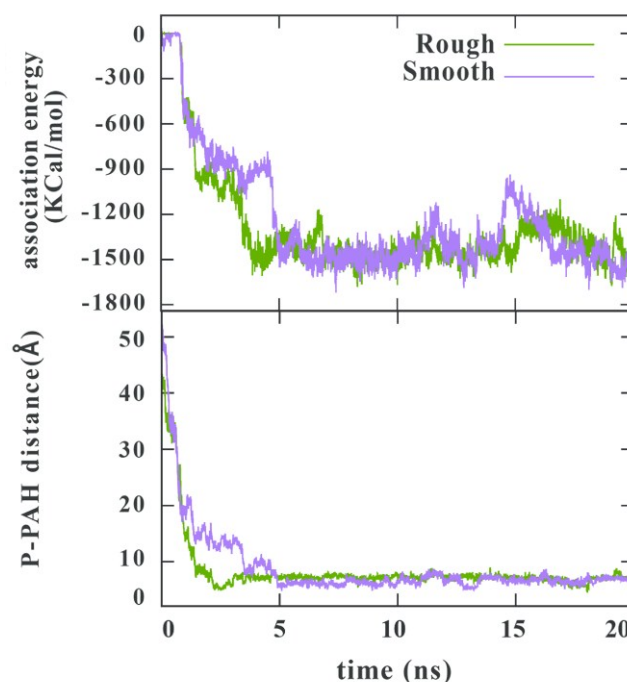


Figure 6. Electrostatic energies of the LPS/PAH association (top) and the distance between a selected phosphorus on LPS to the PAH center of mass (bottom) during a 20 ns simulation of 10-mer PAH interacting with the rough (green) and smoother (yellow) LPS constructs from *P. aeruginosa* shown in Fig. 2. (Note that the electrostatic energy trace for rough LPS is recapitulated from Fig. 5 to facilitate interpretation.)

46 Conclusions

47 This manuscript exploits a set of bacteria which represent a
48 diverse array of environments that nanoparticles may be
49 released into. These bacteria also have important ecological
50 roles, making any effects felt by them impactful on overall
51 environmental health. We demonstrated the use of this
52 bacterial panel in monitoring the toxicity of a model
53 nanoparticle, PAH AuNPs. While we observed increased PAH
54 AuNP binding for bacteria with smooth LPS compared to those
55 with rough LPS, the resulting toxicity did not follow this same
56 trend. We expected that the toxicity observed would correlate
57 with the binding of these NPs to the bacteria, a process
58 mediated by the bacterium's LPS, which is the major surface
59 structure, making up 75% of the Gram-negative bacterial
60 surface for some bacteria.⁴⁷ In reality, the situation is more
61 complex, which demonstrates the importance of using a
62 bacterial panel for nanotoxicity studies. Regardless of the care

1 in controlling for many variables, biology can introduce 53 5
2 complexity to otherwise simple relationships. 54
3 The increased complexity of the biological panel presented 55 6
4 here can be used for several applications. Due to the different 56
5 LPS present on the surface of these bacteria, this panel is a good 57
6 candidate for investigating bacterial surfaces. Indeed, the 58
7 results obtained from the molecular dynamics simulation yield 59 7
8 early insight into the interactions of polyelectrolyte-wrapped 60
9 NPs with bacteria by taking the sugar sequences of the LPS into 61
10 account. This panel is also good for an initial screen of 62
11 nanoparticle toxicity; in using this bacterial panel, we can 63 8
12 identify which bacteria are experiencing an effect specific to 64
13 nanoparticles, which merit further investigation. While we 65 9
14 would expect different results than those presented here if 66
15 different NPs were used, this Gram-negative bacterial panel can 67 10
16 be adapted for use with a range of nanomaterials. 68

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