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Negligible Particle-Specific Antibacterial Activity of Silver Nanoparticles

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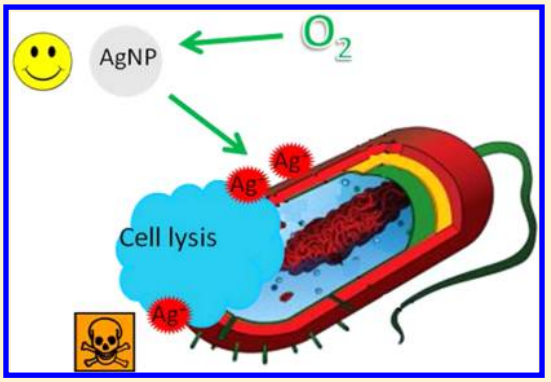
Supporting Information

ABSTRACT: For nearly a decade, researchers have debated the mechanisms by which AgNPs exert toxicity to bacteria and other organisms. The most elusive question has been whether the AgNPs exert direct "particle-specific" effects beyond the known antimicrobial activity of released silver ions (Ag^+). Here, we infer that Ag^+ is the definitive molecular toxicant. We rule out direct particle-specific biological effects by showing the lack of toxicity of AgNPs when synthesized and tested under strictly anaerobic conditions that preclude Ag(0) oxidation and Ag^+ release. Furthermore, we demonstrate that the toxicity of various AgNPs (PEG- or PVP- coated, of three different sizes each) accurately follows the dose–response pattern of *E. coli* exposed to Ag^+ (added as $AgNO_3$). Surprisingly, *E. coli* survival was stimulated by relatively low (sublethal) concentration of all tested AgNPs and $AgNO_3$ (at 3–8 μg/L Ag^+, or 12–31% of the minimum lethal concentration (MLC)), suggesting a hormetic response that would be counterproductive to antimicrobial applications. Overall, this work suggests that AgNP morphological properties known to affect antimicrobial activity are indirect effectors that primarily influence Ag^+ release. Accordingly, antibacterial activity could be controlled (and environmental impacts could be mitigated) by modulating Ag^+ release, possibly through manipulation of oxygen availability, particle size, shape, and/or type of coating.



KEYWORDS: *Silver nanoparticles, toxicity, metallic nanoparticle, silver ion, E. coli*

As a broad-spectrum antimicrobial agent, silver nanoparticles (AgNPs) are currently the most widely commercialized nanomaterial.[1] They are increasingly used in medical and consumer products,[2,3] including household antiseptic sprays and antimicrobial coatings for medical devices that sterilize air and surfaces.[2,4,5]

There is no doubt that the release of silver ions from the crystalline core of silver nanoparticles contribute to the toxicity of these nanomaterials. However, whether the metallic nanoparticle itself exerts a "particle-specific" toxicity remains an elusive question.[6] A specific answer would help both to advance antimicrobial applications of AgNPs and to clarify their potential behavior and impact in the environment. Many toxicity studies using different organisms (e.g., bacteria, algae, fungi, *C. elegans*, zebra fish, *Lolium multiflorum*, human cells) have concluded that the toxicity of AgNPs is not solely due to silver released from the nanoparticle (e.g., silver ions or dissolved silver). In these cases, AgNPs often were more toxic than equivalent concentrations of silver salts.[7−12] However, these observations could be confounded by ligands in the exposure medium that can bind to dissolved silver. These include chloride, sulfide, phosphate, or organic acids whose presence would reduce the bioavailability (and thus the toxicity) of released silver ions to a greater extent than that of AgNPs.[13,14] Other studies have attributed the toxicity of AgNPs solely to dissolved silver, possibly because of the different chemical constituents of the exposure media.[15] In each of these past examples, the studies were conducted under aerobic conditions that promote the continued release of silver ions (Ag^+) from AgNPs and confound the discernment of their relative contribution. Additionally, few studies assessed the residual Ag^+ concentrations (dissolved or adsorbed to the AgNPs coating), which present challenges in differentiating true "particle-specific" toxicity.

Some studies reported that AgNP size,[16−19] shape,[20] surface charge,[21] surface coating,[15] solution chemistry,[13,22] and solubility[15,23] affect AgNPs' toxicity (Table 1). However, the extent to which these factors affected toxicity directly by influencing particle-specific biological effects or indirectly by affecting silver ion release remains an open question. Discerning the relative importance of a particle-specific effect in the antibacterial activity of AgNPs requires careful quantification of the silver ion concentration contributed by the nanoparticle, as well as the role that complexing ligands present in the exposure media could have on silver ion and particle bioavailability.

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Table 1. Proposed AgNPs Toxicity Determinants

property	target organisms	references
size	*E. coli*; human alveolar macrophages	16−19
shape	*E. coli*	20
surface coating	*C. elegans*	15
solution chemistry	Nitrifying bacteria	13
surface charge	*B. subtilis*	21
Ag^+ release	*C. elegans; E. coli, Nitrosomonas europaea*	14, 15, 23, 39
ROS generation	nitrifying bacteria; human monocytic cell; alveolar macrophages; human hepatoma cells; human lung fibroblast cells	7, 19, 40−42

This study aims to resolve these outstanding questions about AgNP toxicity and to determine whether the metallic nanoparticle alone contributes to the antibacterial effects. The work eliminates the possibility of silver ion release by completing some experiments under anaerobic conditions. Additionally, studies are conducted in a minimal medium ($NaHCO_3$ buffer solution, 2 mM) whose constituents have no effect on silver bioavailability (all AgNPs/Ag^+ toxicity assays in this work were below Ag_2CO_3 precipitation potential (K_{sp} = 0.81×10^{-12}, calculated by Visual MINTEC 3.0)). The effects of nanoparticle size and surface coating on antibacterial activity were also considered by synthesizing three types of size-controlled AgNPs to gain insight into particle-specific effects.

Synthesis and Characterization of the PEG-AgNPs. Particle size has frequently been reported to be a determinant of AgNPs' toxicity (Table 1). To confirm this finding, three types of glycol-thiol-coated AgNPs (PEG-AgNPs) were synthesized following the modified procedure as described in Hiramatsu et al.[24] Figure 1 shows that these PEG-AgNPs have well-controlled particles sizes (spherical shape) and narrow size distributions with sizes ranging from 2.8 ± 0.47, 4.7 ± 0.20, to 10.5 ± 0.59 nm. These three types of PEG-AgNPs are referred to in the following text as PEG-3 nm, PEG-5 nm, and PEG-11 nm. These PEG-AgNPs are nonaggregating in both DI water and the minimal medium. The samples remain suspended for at least 6 months inside the anaerobic chamber.

Toxicity of AgNPs Can Be Solely Explained by the Dose−Response of the Released Ag^+. It is well-known that silver nanoparticles can be oxidized in aqueous solutions exposed to air (eq 1) resulting in the release of silver ions under acidic conditions (eq 2)[25]

$$4Ag(0) + O_2 \rightarrow 2Ag_2O \quad (1)$$

$$2Ag_2O + 4H^+ \rightarrow 4Ag^+ + 2H_2O \quad (2)$$

Figure 2 shows that a PEGylation coating on the particles does not protect them against these reactions; under aerobic conditions, silver ion concentrations can be detected and they increase over time (up to 2.1 mg/L after 5 day exposure for PEG-5 nm at pH 4.0). This release pattern could be different and highly variable in the presence of bacteria, which depending on their metabolic state could affect the dissolved oxygen concentration (eq 1), pH (eq 2), release, or remove polymeric substances that coat AgNPs and increase the silver dissolution gradient by binding the released Ag^+. However, no silver ions were released when these AgNPs were stored under anaerobic conditions, suggesting a route for distinguishing the toxicity arising from the nanoparticle with the toxicity arising from the released silver ions. AgNPs that have limited air exposure and whose interactions with microbes are evaluated under strict anaerobic conditions can only impact organisms through particle-specific effects.

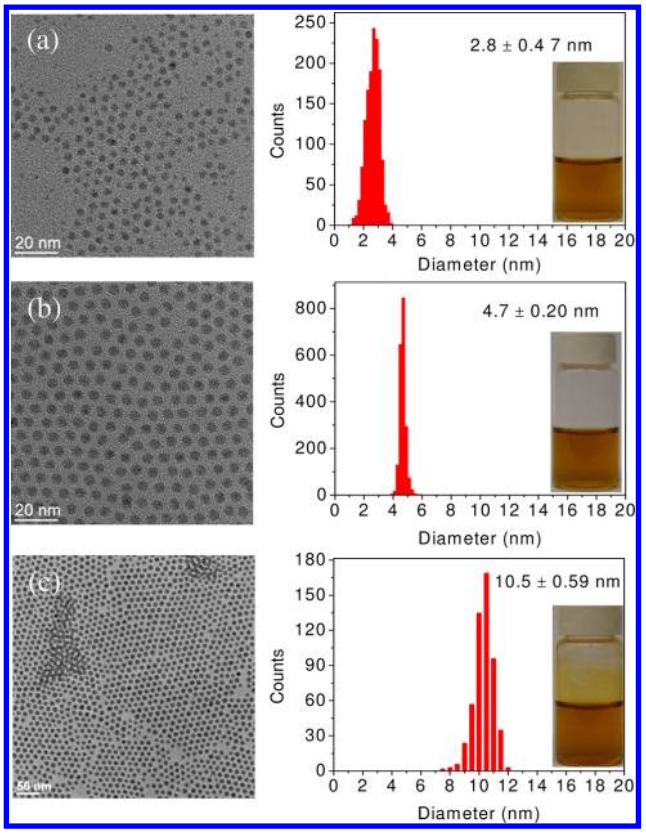

Figure 1. Transmission electron microscopy images and size distribution of the lab synthesized PEG-AgNPs. (a) PEG-AgNPs-3 nm (2.8 ± 0.47 nm); (b) PEG-AgNPs-5 nm (4.7 ± 0.20 nm); and (c) PEG-AgNPs-11 nm (10.5 ± 0.59 nm). The PEG-AgNPs dispersed homogeneously in DI water and showed narrow size distribution, which facilitates the investigation of particles size effect on AgNPs' toxicity. The three insets are pictures of the corresponding suspensions.

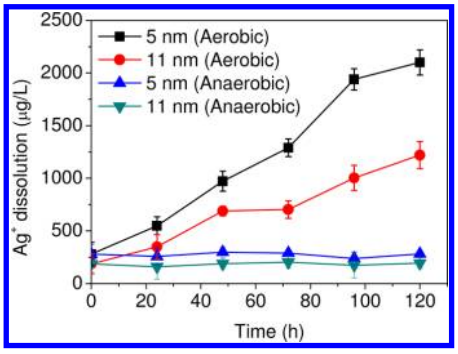

Figure 2. PEG-AgNPs (5 nm and 11 nm) dissolution (at pH 4.0) under aerobic and anaerobic conditions. Dissolved Ag^+ concentration increased with air exposure time for both PEG-5 nm and PEG-11 nm nanoparticles under aerobic conditions, while no silver ions were detected (<1 μg/L) under anaerobic conditions.

To discern the toxicity contribution of the metallic nanoparticles, PEG-AgNPs (PEG-5 and 11-nm) were synthesized and assayed inside the anaerobic chamber. As noted in Figure 2 under such conditions there is no detectable Ag^+ (<1 μg/L). An *E. coli* strain K12 (ATCC 25404) was chosen as a model microorganism for inactivation experiments because it is

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a facultative bacterium that exhibits equal susceptibility to silver ions under aerobic and anaerobic conditions (Supporting Information, Figure S2).[14] Figure 3 showed that under anaerobic conditions the nanoparticles had no measurable effect on *E. coli* up to concentrations thousands of times (6224 and 7665 times) higher than the minimum lethal concentration (MLC) of silver ions themselves (0.025 mg/L) under similar exposure conditions.[14] The lack of toxicity of these nanoparticles at the highest concentrations reached inside the anaerobic chamber (i.e., 158 mg/L for 5 nm PEG-AgNPs and 195 mg/L for 11 nm PEG-AgNPs) suggest that the particles themselves do not affect the biological activity of the microbes. Aerobic toxicity assay using the same anaerobically synthesized PEG-AgNPs were also completed to investigate the confounding effect of the released Ag^+. Toxicity assay (6 h exposure) of PEG-5 nm under aerobic conditions (immediately after transferring out of the chamber) showed enhanced toxicity, indicating that silver ion released during the toxicity assay can have a notable antimicrobial effect. Prolonged air exposure (48 h with magnetic stirring to increase oxygen exposure) induced higher antibacterial toxicity of the PEG-5 nm silver nanoparticles. These results illustrate that the toxicity of AgNPs is very sensitive to the presence of air. Oxidative dissolution of the crystalline cores can result under aerobic conditions and increase the concentration of soluble silver ions.

We speculated that all of the aerobic toxicity of the silver nanoparticles could be explained by the presence of released Ag^+. To test this hypothesis, air-exposed PEG- and PVP-AgNPs (commercially available, Supporting Information, Figure S1) suspensions were tested for antimicrobial activity. These assays were conducted inside an anaerobic chamber to stop the increased dissolution of particles during the time scale of the experiments. Figure 4 shows that the antimicrobial activity of all AgNPs when expressed in terms of the measured concentrations of silver ion were statistically indistinguishable ($p > 0.05$, Supporting Information, Table S2) from the toxicity of silver ions introduced through silver nitrate. The fact that the dose–response patterns of the six different AgNPs could be explained by the concentration of the released Ag^+ corroborates that the antimicrobial activity was solely due to the released Ag^+ and that no direct particle-specific effects contributed to toxicity.

For nearly a decade, researchers have debated the mechanisms by which AgNPs exert toxicity to bacteria and other organisms (especially whether the AgNPs exert direct "particle-specific" toxicity). These results demonstrate that the antimicrobial activity of AgNPs is solely due to Ag^+ release and that even relatively low (μg/L) concentrations of Ag^+ (released or adsorbed to AgNP coatings) can account for the biological response observed in previous studies. Particle properties that affect toxicity such as size,[16−19] shape,[20] surface coating,[15] and surface charge[21] likely affect toxicity indirectly through mechanisms that influence the rate, extent, location, and/or timing of Ag^+ release. For example, AgNPs of smaller size may exert higher toxicity due to their higher specific surface area and associated faster Ag^+ release rate compared to larger AgNPs.[17] To fully control AgNPs toxicity will require deeper understanding of the release, speciation, and bioavailability of Ag^+.[25,26]

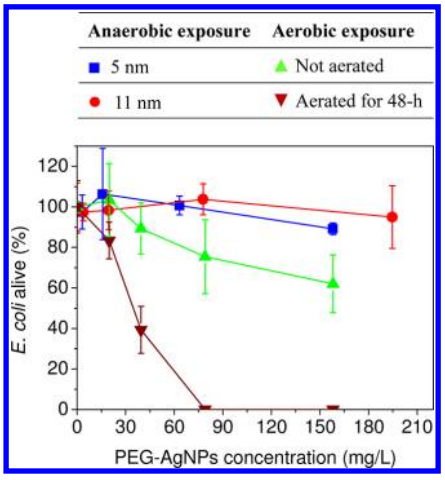

Figure 3. Elimination of toxicity by AgNP synthesis and exposure under anaerobic conditions that preclude oxidative Ag^+ release (less than 1 μg/L, by ICP-MS). Viability assays show no statistically significant toxicity with concentration up to 158 (for 5 nm AgNPs) and 195 mg/L (for 11 nm AgNPs), which were the highest concentration reached inside the anaerobic chamber. These nonlethal concentrations are respectively 6224 and 7665 times higher than MLC for Ag^+, indicating negligible toxicity. Antibacterial assays (6 h exposure) with the same 5-nm PEG-AgNPs under aerobic conditions (conducted immediately after transferring the particles out of the chamber) showed toxicity, illustrating the potential confounding effect of Ag^+ release during exposure. Storage in an aerobic atmosphere (48 h with magnetic stirring to increase oxygen exposure) resulted in higher Ag^+ release and higher toxicity.

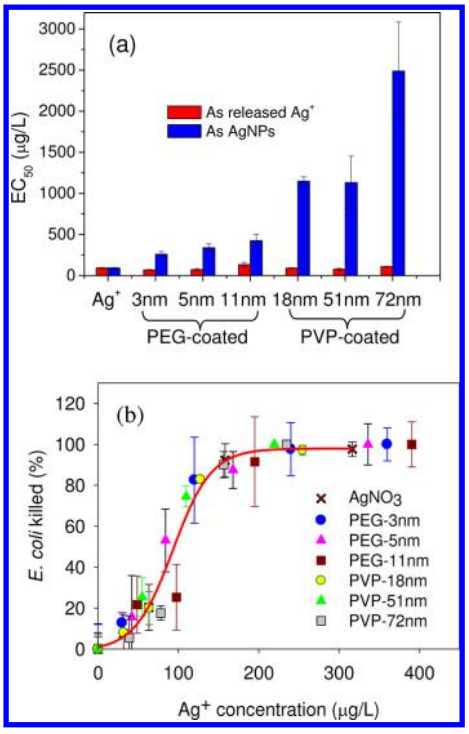

Figure 4. Dose–response of *E. coli* exposed to various air-exposed AgNPs. EC_{50} increases with the increasing particle size (a), suggesting size-dependent toxicity. This is an indirect effect associated with Ag^+ release (smaller AgNPs release more Ag^+ and are more toxic). Antibacterial activity expressed as a function of the concentration of the released Ag^+ was statistically indistinguishable from the dose response patterns of cells exposed to Ag^+ (added as $AgNO_3$) ($p > 0.05$), illustrating that the released Ag^+ is the critical factor of antibacterial activity (b).

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***E. coli* Survival Was Stimulated by Low Silver Doses.** Survival of resting *E. coli* cells in 2 mM $NaHCO_3$ buffer solution was stimulated in the presence of low Ag^+ concentrations with 13% higher bacteria viability in the $AgNO_3$-treated group after 6 h exposure than in the unexposed control group (Figure 5a).

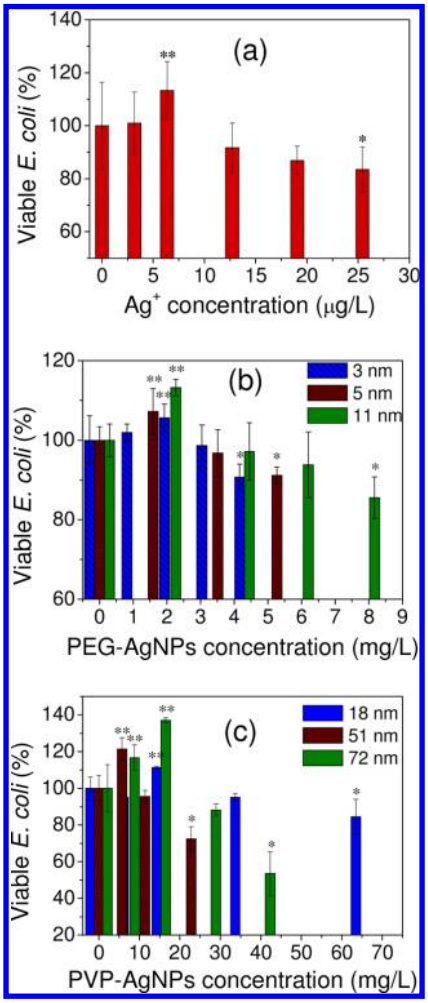

Figure 5. Survival of resting *E. coli* cells in 2-mM $NaHCO_3$ buffer solution after 6 h exposure to (a) $AgNO_3$, (b) PEG-AgNPs, and (c) PVP-AgNPs. One asterisk represents significant decrease in viability ($p < 0.05$) relative to unexposed control and corresponds to the minimum lethal concentration (MLC). A significant stimulatory effect suggestive of hormesis was observed at some sublethal concentrations of all treatments, as indicated by two asterisks.

This enhanced tolerance phenomenon was also observed as a result of exposure to lower concentrations of all tested AgNPs with higher survival rates of 6% for PEG-AgNPs-3 nm at 2.2 mg/L, 7% for PEG-AgNPs-5 nm at 1.8 mg/L, and 13% for PEG-AgNPs-11 nm at 2 mg/L (Figure 5b) and 11% for PVP-AgNPs-20 nm at 16.4 mg/L, 21% for PVP-AgNPs-40 nm at 5.7 mg/L and 17% for PVP-AgNPs-80 nm at 6.7 mg/L (Figure 5c). This apparent hormetic effect[27,28] might have been triggered by the residual Ag^+ in air-exposed AgNPs stock suspensions (Supporting Information, Table S1) that could not be completely separated by filtration. The residual Ag^+ concentrations in AgNPs stock suspensions ranged from 0.28 mg/L (in PEG-AgNPs-11 nm stock suspensio, 82.0 mg/L) to 7.9 mg/L (in PVP-AgNPs-40 nm stock suspension, 5700 mg/L), corresponding to Ag^+ concentration of 3–7.9 μg/L (at the tested AgNPs doses), or 12–31% of the MLC for Ag^+. This finding suggests that sublethal concentrations of silver may enhance bacterial fitness and hinder antimicrobial applications.

A hormesis effect has also been observed in studies of the impact of silver nanoparticles toxicity studies on human cell lines (e.g., peripheral blood mononuclear cells, human hepatoma derived cell line HepG2).[22,29−31] Moreover, other kinds of nanoparticles, that is, carbon nanotubes,[32] quantum dots,[33,34] and metal nanoparticles,[35] also have shown a stimulatory effect at sublethal exposures. Apparently, the presence of low doses of toxicants can activate repair mechanisms of the cells against the toxicant, and this repair process may sometimes overcompensate for the exposure.[36] The poorly understood hormetic response of Ag^+ on *E. coli* cells and other nanomaterials on all kinds of organisms underscores the need for further study of its responsible mechanisms.

Implications for AgNPs Antibacterial Application and Environmental Impact. Whereas AgNPs themselves do not significantly exert direct particle-specific toxicity on bacteria, AgNPs could be engineered with different particle formations (e.g., surface coatings) to release Ag^+ at desired rate and location. Furthermore, AgNPs may serve as a vehicle to deliver Ag^+ more effectively (being less susceptible to binding and reduced bioavailability by common natural ligands[14]) to the bacteria cytoplasm and membrane (Figure 6), whose proton motive force would decrease the local pH (as low as pH 3.0)[37,38] and enhance Ag^+ release (Supporting Information, Figure S3).

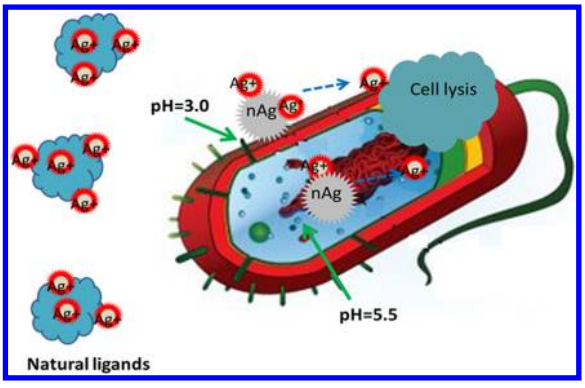

Figure 6. Schematic of AgNPs, Ag^+, and cell interactions. AgNPs may serve as a vehicle to deliver Ag^+ more effectively (being less susceptible to binding and reduced bioavailability by common natural ligands) to the bacteria cytoplasm and membrane, whose proton motive force would decrease the local pH (as low as pH 3.0) and enhance Ag^+ release.

Although this research was conducted with a model bacterium (*E. coli*), our approach to separate the contributions of Ag^+ from AgNPs may benefit the etiology of AgNP toxicity to higher order organisms (e.g., algae, zebra fish, *C. elgans*). In these cases, however, organism-specific immune responses to different nanoparticle morphologies could lead to different observations, underscoring the need for caution when extrapolating our mechanistic inferences to other biological systems. Our approach to separate exposure to nanoparticles in the absence of dissolved metal may also be used to advance mechanistic understanding of the bacterial toxicity exerted by other metal-based NPs (e.g., CuO, ZnO, QDs) that also release toxic metal ions.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental methods for AgNPs characterization, *E. coli* growth inhibition assay, anaerobic PEG-AgNPs synthesis, air-exposed AgNPs preparation, AgNPs filtration, statistical analysis, Figures S1,S2, Tables S1,S2, and corresponding discussions are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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