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Surface Charge-Dependent Toxicity of Silver Nanoparticles

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As a result of the extensive number of applications of silver nanoparticles (AgNPs), their potential impacts, once released into the environment, are of concern. The toxicity of AgNPs was reported to be dependent on various factors such as particle size, shape and capping agent. Although these factors may play a role in AgNPs toxicity, the results presented herein suggest that surface charge is one of the most important factors that govern the toxicity of AgNPs. In the current study, the toxicity of four AgNPs representing various surface charging scenarios ranging from highly negative to highly positive was investigated. These AgNPs were (1) uncoated H_2−AgNPs, (2) citrate coated AgNPs (Citrate-AgNPs), (3) polyvinylpyrrolidone coated AgNPs (PVP-AgNPs), and (4) branched polyethyleneimine coated AgNPs (BPEI-AgNPs). Our results clearly demonstrate that the AgNPs exhibited surface charge-dependent toxicity on the *bacillus* species investigated. Furthermore, ultrafiltration membranes were utilized to purify the AgNPs suspensions from residual impurities prior to the introduction to the microbes. This step was crucial in determining the true AgNPs toxicity and is either missing or not explicitly mentioned in most of the reported toxicity studies.

1.0. Introduction

Evidence from several studies has revealed that silver nanoparticles (AgNPs) may inherit the antimicrobial properties associated with ionic silver (Ag^+) (*1*−*3*). This resulted, in an exponential increase in the manufacturing and use of AgNPs for disinfection purposes in a wide spectrum of consumer products such as fabrics, plastics, papers, and washing machines (*4*−*6*). The unique optical and physical properties of AgNPs have also led to their use in many other applications such as catalysis, electronics, surface enhanced Raman Spectroscopy (SERS) and sensing applications (*6*). Because of the extensive applications of AgNPs, there are concerns with the potential for detrimental environmental impacts once released into the environment (*4, 7*).

Various research studies have investigated the toxicity of AgNPs on a range of organisms, mammalian, and human cells (*8*−*10*). Although, the exact toxicity mechanisms of AgNPs are still unclear, many proposed mechanisms can be found in the literature. These mechanisms include, dissolution of Ag^+ from the AgNPs (*11, 12*), association of AgNPs with cell membranes that might cause physical damage (e.g., pitting) or the subsequent penetration that cause cell malfunction (*13, 14*), stimulation of reactive oxygen species and interaction with specific proteins and/or enzymes inhibiting their activities (*15, 16*).

Even though research has been unclear with regards to the toxicity mechanisms of AgNPs, research has shown a toxicity dependence on factors such as particle size (*17*), shape (*18*) and surface properties (*12, 19*). In spite of the plethora of AgNPs toxicity studies, there still is a high level of uncertainty with regards to the true toxicity of AgNPs. This may be partially attributed to the presence of impurities (e.g., Ag^+, residual reducing and stabilizing agents from AgNPs synthesis) in the tested AgNPs suspensions (*6, 11*). Even though purification techniques such as dialysis and ultrafiltration are available (*20*), the use of these techniques has not been explicitly presented in most of the methodologies presented in the literature (Supporting Information (SI) Table S1). Most AgNPs suspensions contain unquantified Ag^+ impurities as a result of the incomplete reduction of the silver salt precursors during synthesis (*11*).

The current study aimed at utilizing the five day oxygen consumption test and the live/dead technique as rapid screening methods for evaluating the toxicity of four different purified AgNPs suspensions on *bacillus* species (Gram-positive bacteria). The evaluated AgNPs were selected in order to represent various surface charging scenarios ranging from high negative to high positive.

2.0. Experimental Section

2.1. Synthesis and Purification of AgNPs Suspensions. The four AgNPs investigated in the current study were (1) uncoated H_2−AgNPs, (2) citrate coated AgNPs (Citrate-AgNPs), (3) polyvinylpyrrolidone coated AgNPs (PVP-AgNPs), and (4) branched polyethyleneimine coated AgNPs (BPEI-AgNPs). The H_2−AgNPs, Citrate-AgNPs and BPEI-AgNPs suspensions were prepared as described in our previous research (*21*). The PVP-AgNPs was prepared according to Lee and Meisel (*22*) with slight modifications (details of synthesis are provided in the SI). The H_2−AgNPs stabilization mechanism is also presented in the SI. The purification of the AgNPs suspensions was performed using a system purchased from Spectrum Laboratories, Inc. (schematic provided in SI Figure S1). The ultrafiltartion membrane used was 10 kDa polyethersulfone (PES) (MidiKros Hollow Fiber Module (P-X3-010E-300-02N). Conductivity was used as an indicator for the presence of impurities in the synthesized AgNPs suspensions. Lower conductivity was translated into less impurity in the suspensions (see SI Table S2). Milli-Q water (conductivity 0.67 μs cm^{-1}) was used to wash free Ag^+ ions as well as other impurities from the AgNPs suspensions. The retentate conductivity was continuously monitored until achieving a conductivity of less than 10 μs cm^{-1} (Orion 013010MD conductivity cell). Initial and final suspension conductivities are presented in Table 1. The conductivity of the purified AgNPs suspensions was further measured periodically for 3 weeks after the purification process in order to evaluate the release of Ag^+ from AgNPs. The purity of the AgNPs suspensions was verified using X-ray photoelectron

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TABLE 1. Characteristics of AgNPs before and after Purification

parameter		as prepared (unpurified)				immediately after purification				three weeks after purification			
		H_2−AgNPs	citrate-AgNPs	PVP-AgNPs	BPEI-AgNPs	H_2−AgNPs	citrate-AgNPs	PVP-AgNPs	BPEI-AgNPs	H_2−AgNPs	citrate-AgNPs	PVP-AgNPs	BPEI-AgNPs
HDD (nm)[a]	size	18	10	12	10	18	10	12	10	17	11	12	10
	% volume	97.5	98.5	98.3	96.7	98.3	99.0	96.8	98.0	98.6	98.9	99.5	97.8
	PW[b]	15.2	8.4	5.2	2.8	13.5	6.7	5.4	3.4	17.2	5.4	6.9	3.7
ζ potential[c] (mV)		−22	−38	−10	+40	−22	−40	−12	+39	−23	−38	−10	+41
conductivity (μs cm^{-1})		65	550	350	1005	4.8	9.8	5.4	8.4	4.4	8.9	5.7	8.0

[a] Hydrodynamic diameter (volume distribution) measured at pH 7.0. [b] Peak width at half-maximum. [c] Measured zeta potential at pH 7.0.

spectroscopy (XPS) and nuclear magnetic resonance (NMR) techniques (details are presented in the SI).

2.2. Characterization of AgNPs Suspensions and Bacterial Cells. The hydrodynamic diameter (HDD) and zeta (ζ) potential of the AgNPs were measured using a Zetasizer Nanoseries (Malvern Instruments). Measurements were taken before and after purification and intermittently for 3 consecutive weeks. Total Ag concentration was measured using a PerkinElmer AAnalyst 800 atomic absorption spectrometer after microwave acid digestion following EPA method 3015A. Transmission Electron Microscopy (TEM) was used to acquire micrographs for both the AgNPs and the bacterial cells. A JEOL-1200 EX TEM (JEOL Inc., USA) was used for imaging the AgNPs and Phillips Biotwin 12 (FEI Company, USA) TEM was used to acquire the bacterial cell images in the presence and absence of AgNPs.

2.3. Oxygen Consumption. Standard biological oxygen demand (BOD_5) method 2510 B (*23*) was used to determine the oxygen consumption as a microbial activity indicator. The BOD_5 test media was prepared by adding 1 mL each of phosphate buffer, $MgSO_4$, $CaCl_2$, and $FeCl_3$ solutions to a liter of Milli-Q water. The test media was aerated until saturation with oxygen was reached. The detailed composition of the above-mentioned solutions is provided in the SI. The tested organisms were PolySeed (Gram-positive *bacillus* species) obtained from, Interlab Supply. Each concentration of ionic and metallic forms of Ag was tested in triplicate. Silver nitrate ($AgNO_3$) was used as the source for Ag^+. A mixture of glucose and glutamic acid (3 mg L^{-1} each) was used as a positive control and the same amount of glucose and glutamic acid mixture was added to each sample. The dissolved oxygen (DO) concentrations were measured using a Thermo Scientific Orion DO probe immediately after the introduction of treatments as well as after 120 ± 2 h. The oxygen consumption was calculated as the difference between these two measurements.

2.4. Microbial Live/Dead Measurements. A live/dead *BacLight* kit with two color fluorescence assay coupled with fluorescence spectroscopy was used to perform bacterial viability (Molecular Probes Inc.). Component A (SYTO 9), and component B (Propidium iodide) from the *BacLight* kit were mixed in a 1:1 (v/v) ratio. Three microliters of this mixture was added to 1 mL of each sample in a microfuge tube and incubated for 15 min in the dark at room temperature. After the incubation, fluorescence emission spectrum (470−700 nm) of each sample was recorded at 470 nm excitation using a spectrofluorophotometer (Shimadzu RF-5301PC). Prior to sample measurement a calibration curve was constructed using ratios of live/dead bacteria. The different percentage of live bacteria (0, 25, 50, 75, and 100) was achieved using 70% isopropyl alcohol. The ratio of the integrated intensity of the wavelength range between 510 and 540 nm (for live; green) to that of 620−650 nm (for dead; red) was calculated. Duplicate samples were tested from each treatment directly after DO measurements at the end of the incubation period. Moreover, the spectra of the fluorescent dyes were unaffected by the utilized capping agents (SI Figure S2).

3.0. Results and Discussion

A detailed characterization of the investigated AgNPs is described in our previous research (*21*). Nevertheless, the basic characteristics that might contribute to the toxicological behavior of these AgNPs (e.g, the hydrodynamic diameter (HDD) and the zeta (ζ) potential) are summarized in Table 1. As mentioned earlier, the toxicity of silver nanoparticles is dependent on many factors. Some of these factors are the presence of impurities, the particle shape, size and surface properties.

3.1. Impact of Impurities. It is crucial that the AgNPs suspension purification removes the impurities without affecting the characteristics of the NPs. Impurities present in AgNPs suspensions do not only include Ag^+ but also the residual reducing and stabilizing agents from the synthesis process. As presented in Table 1, the similarities in the measured characteristic parameters, before and after purification, suggest that the purification process did not impact the characteristics of these AgNPs. In order to assess the efficiency of the purification process, the reduction in the suspension's conductivity was used as an indicator for the removal of impurities. The reduction in impurities was further confirmed by X-ray Photoelectron Spectroscopy (XPS) and proton Nuclear Magnetic Resonance (^{1}H NMR) analyses results (details are provided in SI Figures S3−S5). Furthermore, the XPS analysis showed that the investigated AgNPs were at the Ag^0oxidation state. This excludes any difference in the toxicity of the studied AgNPs as a result of the oxidation state of the AgNPs.

The removal of the residual impurities had a dramatic impact on the toxicity of AgNPs. The toxicity of two AgNPs suspensions (Citrate-AgNPs and H_2−AgNPs) was evaluated before and after purification (SI Figure S6). The observed reduction in toxicity is most likely caused by the removal of Ag^+ from the AgNPs suspension. Furthermore, the utilized capping agents themselves (citrate, PVP and BPEI) did not show any measurable toxicity since there was no reduction in oxygen consumption when compared to the positive control (data are presented in SI Table S3). Toxicity of a mixture of capping agent and Ag^+ was also evaluated and the results showed no synergetic effects with Ag (SI Table S3).

Even after purification, the dissolution of AgNPs to Ag^+ can be a source of toxicity. The constant conductivity of the purified AgNPs suspensions, during the 3 weeks experiment period (Table 1), might imply minimal dissolution of AgNPs into Ag^+. The 3 weeks period, during which the AgNPs suspension conductivity was monitored, was the total time elapsed between the particle synthesis, purification and the conclusion of the toxicity test. The results presented here diminish the likelihood of significant toxicological impacts associated with any impurities as well as capping agents in the investigated AgNPs suspensions.

3.2. Impact of Shape and Size. As previously mentioned, research suggests that the shape and size of AgNPs can impact the toxicity of these particles (*17, 18*). The AgNPs investigated were all spherical (transmission electron micrographs are provided in the SI Figure S7) which excludes particle shape as a contributing factor to toxicity in the current study. The HDD of the investigated AgNPs ranged from 10 to 18 nm (Table 1), well below the size at which nanoparticles exhibit their unique properties (*24*). Additionally, the neutral pH and low ionic strength (3 mM) of the test media do not allow the investigated AgNPs to aggregate (*21*) which allows for a more accurate toxicity evaluation (*25*). This was confirmed by measuring the HDD of the investigated AgNPs diluted in BOD test media (see SI Table S4).

The toxicity of the investigated AgNPs on *bacillus* species varied as demonstrated by the oxygen consumption and the percent live bacteria (Figure 1). While Citrate-AgNPs and BPEI-AgNPs had similar average HDD (10 nm), they provided two drastically different toxicity profiles. Furthermore, H_2−AgNPs (18 nm), was expected to have the least toxicity among the AgNPs investigated based on particle size alone as previous research showed a decrease in toxicity with an increase in particle size of AgNPs (*17*). While BPEI-AgNPs (10 nm), as expected, exhibited higher toxicity than the larger H_2−AgNPs (18 nm), Citrate-AgNPs exhibited lower toxicity (Figure 1). Based on these conflicting observations, it appears that particle size was not the dominant factor in determining

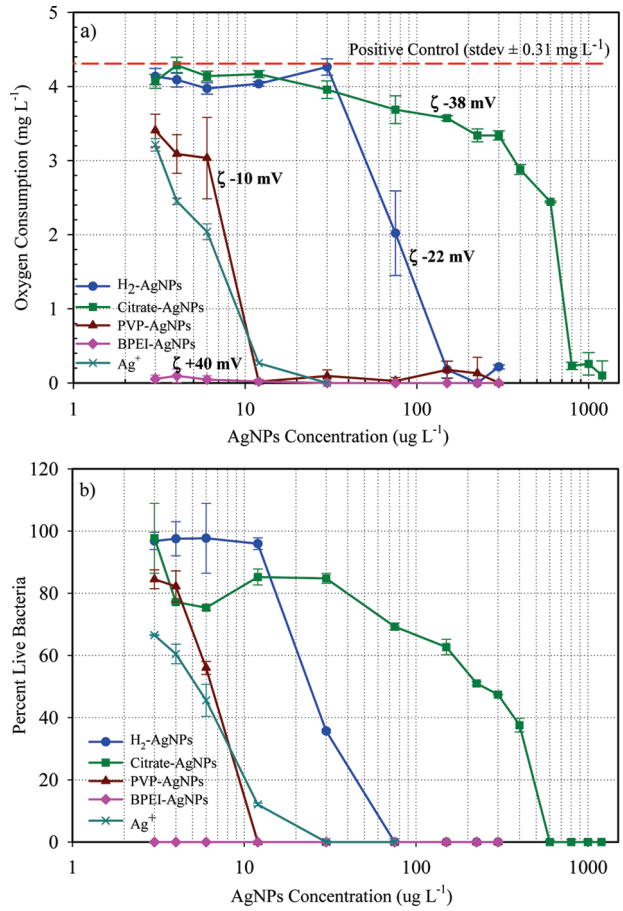

FIGURE 1. AgNPs toxicity shows correlation with surface charge. (a) Oxygen consumption after 5 days. (b) Percent live bacteria after 5 days.

the toxicity in the current study. Nevertheless, particle size may still influence the toxicity of AgNPs under other testing conditions and/or if NPs with same capping agent but different particle sizes were utilized.

3.3. Surface Charge-Dependent Toxicity. The ζ potential, which reflects the charge on the AgNPs surface, varied in type (negative/positive) as well as the magnitude based on the capping agent or the reactants used during the synthesis process (Table 1). Results presented here show a direct correlation between the toxicity of AgNPs and their surface charge. The more negative Citrate-AgNPs were the least toxic, whereas the positively charged BPEI-AgNPs were the most toxic NPs as presented in Figure 1. The ionization of the citrate molecules, coating the Citrate-AgNPs, has resulted in a ζ potential of −38 mV which is similar to that of the tested *bacillus* species (−37 mV under test conditions). The carboxyl, phosphate and amino groups on the cellular membrane of the Gram-positive bacteria provide the organisms with a negative charge (*26*). Thus, there is a high degree of repulsion, between the negatively charged Citrate-AgNPs and the *bacillus* cells, which forms an electrostatic barrier that limits the cell-particle interactions thus reducing the toxicity. As the magnitude of the negative ζ potential gradually decrease, H_2−AgNPs (−22 mV) and PVP-AgNPs (−10 mV), the electrostatic barrier is reduced which increases the chances of cell-particle interactions and results in higher toxicity. The repulsion turned to attraction when the bacteria were exposed to the positively charged BPEI-AgNPs (+40 mV). This allows for a higher degree of interactions which causes a greater toxicity.

It appears that the mechanisms of AgNPs toxicity may involve a combination of both physical and chemical

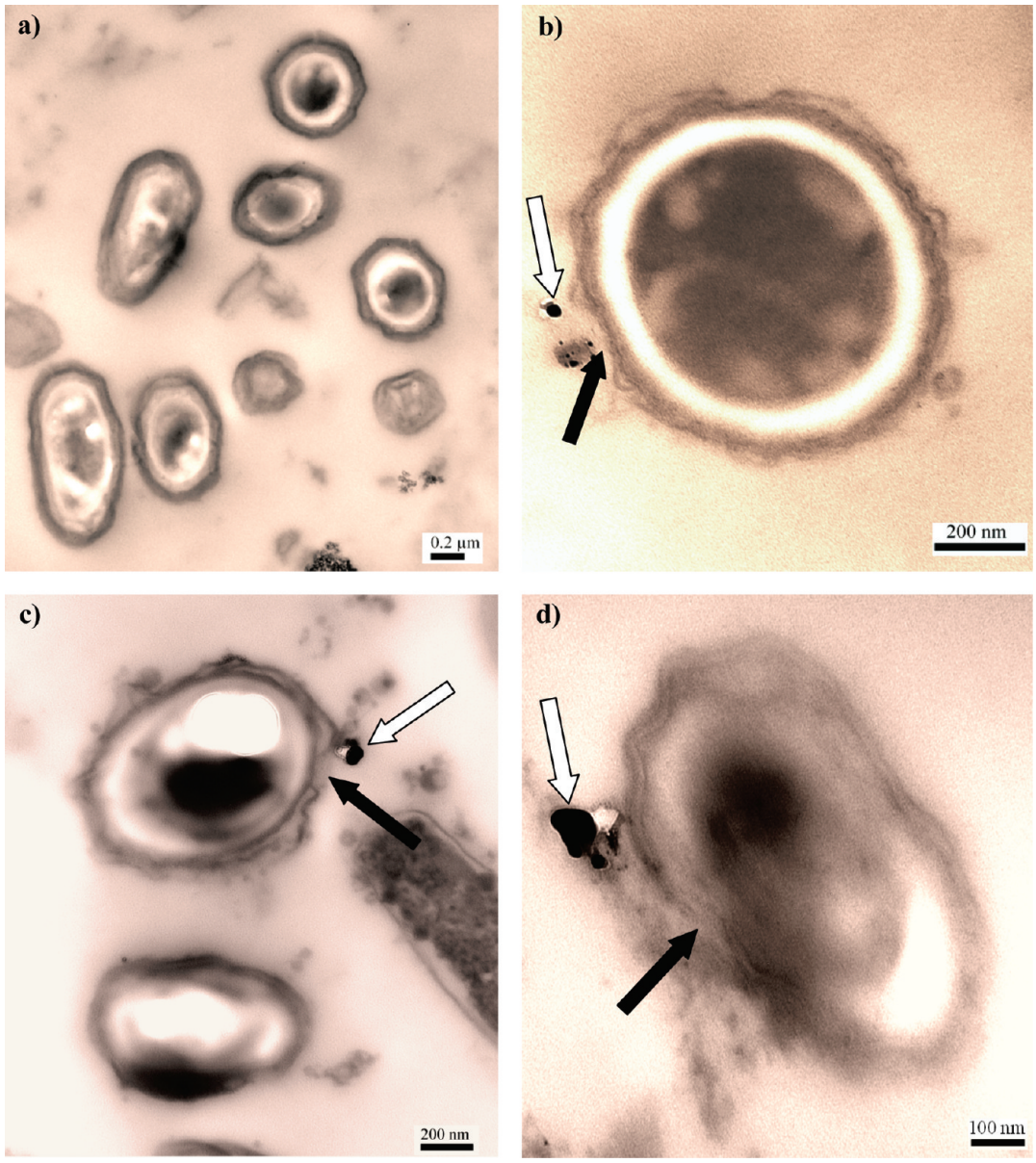

FIGURE 2. Transmission electron micrographs (TEM). (a) unexposed (control) cells. (b−d) exposed cells to BPEI-AgNPs, white arrows refer to the AgNPs and black arrows refer to the impacts on the cellular membranes.

interactions with the former one as the limiting step. Once the electrostatic barrier is overcome, the AgNPs can interact with the cellular membrane and either cause physical damage (i.e., pitting (*27*)) that ultimately leads to cellular death or pass through the membrane and, for example, chemically induce reactive oxygen species (*15*). The TEM micrographs presented in Figure 2 suggest that, under the conditions of the current study, the physical interactions may have played a major role in rupturing the cellular membrane (more TEM micrographs are presented in SI Figure S8). Furthermore, the significantly higher toxicity of BPEI-AgNPs when compared to the also positively charged Ag^+ supports the assumption that physical interaction, damaging the cellular membrane, is the primary mechanism for AgNPs toxicity when compared to the only chemical effect caused by the Ag^+ alone (*28*). These findings may be limited to the *bacillus* species investigated. Other bacteria may be more resistant to the physical interactions encountered here.

4.0. Significance

Many factors can impact the toxicity of AgNPs. However, results presented herein demonstrate the importance of the physical interactions, between the AgNP and the bacterium, on the toxicity of AgNPs. These physical interactions are highly governed by the surface charge of not only the AgNPs but also of the cellular membranes of the bacteria examined. Although, in this study, particle shape and size had minimal or no influence on the toxicity of the evaluated AgNPs, we do not exclude these factors from having an impact on the toxicity of AgNPs in the natural environment. Nonetheless, we believe that unless the electrostatic barrier between the AgNPs and the bacteria is overcome, other toxicity factors (i.e., shape and size) may have minimal impact on the toxicity of these nanoparticles. As a result, it is in our opinion that surface charge is one of the most important factors that has to be taken into consideration when evaluating the toxicity of AgNPs in the environment. In addition, we expect that surface charge-dependent toxicity behavior might eventually be a good tool for the prediction of the toxicological behavior of various types of AgNPs. Furthermore, our research demonstrates the importance of the removal of impurities from nanoparticles suspensions on the accurate evaluation of toxicity.

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Supporting Information Available

Summary for several toxicity studies of AgNPs, the purification system used to remove impurities from AgNPs suspensions, XPS and NMR methodologies and results, toxicity data for unpurified AgNPs suspensions and TEM for the utilized AgNPs. This material is available free of charge via the Internet at http://pubs.acs.org.

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