Anaerobic Toxicity of Cationic Silver Nanoparticles

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Abstract

The microbial toxicity of silver nanoparticles (AgNPs) stabilized with different capping agents was compared to that of Ag⁺ under anaerobic conditions. Three AgNPs were investigated: (1) negatively charged citrate-coated AgNPs (citrate-AgNPs), (2) minimally charged polyvinylpyrrolidone coated AgNPs (PVP-AgNPs) and (3) positively charged branched polyethyleneimine coated AgNPs (BPEI-AgNPs). The AgNPs investigated in this experiment were similar in size (10-15nm), spherical in shape, but varied in surface charge which ranged from highly negative to highly positive. While, at AgNPs concentrations lower than 5 mgL⁻¹, the anaerobic decomposition process was not influenced by the presence of the nanoparticles, there was an observed impact on the diversity of the microbial community. At elevated concentrations (100 mgL⁻¹ as silver), only the cationic BPEI-AgNPs demonstrated toxicity similar in magnitude to that of Ag⁺. Both citrate and PVP-AgNPs did not exhibit toxicity at the 100 mgL⁻¹ as

measured by biogas evolution. These findings further indicate the varying modes of action for nanoparticle toxicity and represent one of the few studies that evaluate end-of-life management concerns with regards to the increasing use of nanomaterials in our everyday life. These findings also highlight some of the concerns with a one size fits all approach to the evaluation of environmental health and safety concerns associated with the use of nanoparticles.

Keywords: silver nanoparticles, anaerobic digestion, dissolution, stabilization mechanism, anaerobic toxicity.

1.0 Introduction

Nanotechnology has seen a dramatic increase in utilization in environmental, medical, chemical and pharmaceutical industries (1). Engineered nanoparticles (ENPs) possess unique characteristics, compared to their bulk counterparts, such as the large surface area to volume ratio, high chemical reactivity, unique antimicrobial/ fungicidal activity, and biocompatible surface properties (2). Most of these properties are directly attributed to the small particle size of nanomaterials and only manifest within a particular size regime (3). These unique properties of materials at the nanoscale raise the need to examine the behavior of these particles in diverse environmental scenarios. Fundamental research on the fate, transport and toxicity of NPs is essential within an effort to determine their impact on natural and engineered environmental systems such as groundwater, soils and sediments, wastewater treatment and municipal solid waste management systems.

In particular, silver which has been well known for its antibacterial properties for centuries has become among the most commercially used nanoparticles (4). Currently silver nanoparticles are employed in many consumer products such as textiles, biomedical products, plastics, socks, food storage containers and various cleaning products (5). These particles are synthesized using various techniques to produce particles with different chemical and physical characteristics (6). Most of the AgNPs incorporated into consumer products are coated, surface modified and or functionalized to achieve certain properties (7, 8). Different surface functionalization, obtained by applying different capping agents, was shown to influence the toxicity, aggregation and dissolution of AgNPs (9, 10).

A considerable amount of silver may be released from silver nanoparticle-containing consumer products within just a few washing cycles (11, 12). Once released, depending on the circumstances, AgNPs can end up in a variety of environmental scenarios. It is highly likely that AgNPs travel through municipal sewer lines and reach wastewater treatment plants (WWTPs) and potentially accumulate in the biosolids (13-15).

One of the primary methods of biosolids treatment utilized is anaerobic digestion which relies on active anaerobic bacterial communities to degrade the organics (16). Therefore, it is vital to assess the potential impacts AgNPs may have on these bacterial species under anaerobic conditions and also the possible transformations of these particles within these systems. In addition to aggregation, AgNPs may undergo surface changes that influence their behavior. For example, in a system with elevated chlorides and sulfides levels (e.g., wastewater, composter leachate and landfill leachate), the released AgNPs may transform to silver chlorides (AgCl) and silver sulfides (Ag₂S) which are less toxic relative to ionic Ag and metallic AgNPs (17-19).

It is worthy to mention that the antibacterial properties of AgNPs on microorganisms under aerobic conditions have been significantly studied (20). The antibacterial mechanism of silver nanoparticles is linked to a combination of release of Ag^+ by AgNPs oxidative dissolution or specific nanoparticle properties such as Trojan-horse type mechanism and generation of reactive oxygen species (ROS) leading to cell membrane damage (15, 21). Comparatively, the antibacterial activity of AgNPs under anaerobic conditions has been less studied and understood. According to Kim et al., 2010, municipal wastewater treatment plants control the flows of silver between anthropogenic and environmental compartments (18). Therefore, the current study aims at investigating the effect of AgNPs on the anaerobic degradation process in simulated environmental systems. The antibacterial impacts of different AgNPs on the digestion process of anaerobic biosolids were studied at various concentrations and compared to that of Ag^+ . Based on the observed surface charge dependent impacts, the nanoparticles exhibiting toxicity were further investigated by performing real-time speciation analysis and taxonomical analysis.

2.0 Experimental Section

2.1 Nanoparticles Synthesis, Purification, and Characterization

Three types of AgNPs with different capping agents were utilized, citrate coated AgNPs (citrate-AgNPs), Polyvinylpyrrolidone coated AgNPs (PVP-AgNPs), and branched polyethyleneimine-coated AgNPs (BPEI-AgNPs). The nanomaterials were prepared and purified as described by El Badawy et al. (22). The hydrodynamic diameter (HDD) and zeta potential (ζ) of the AgNPs were measured using a Zetasizer Nanoseries (Malvern Instruments). Transmission electron microscopy (TEM) was used to verify nanoparticles' size and shape. TEM samples were prepared by depositing a drop of the particular nanoparticle suspension on a carbon coated copper grid. Samples were air-dried at room temperature overnight in a dust-free box. Images were captured

using a JEOL-1200 EX TEM (JEOL Inc.) operated at 120 kV. The total Ag concentration of the suspensions was measured using a PerkinElmer AAnalyst 800 atomic absorption spectrometer after performing a microwave acid digestion following EPA method 3015A.

2.2 Anaerobic Digesters Setup

Anaerobic biosolids were collected from a wastewater treatment plant. The characteristics of the biosolids are presented in Table S1 in supporting information (SI). In an anaerobic chamber, 30 serum bottles (250 mL total volume) were each filled with 120 mL of biosolids, sealed, covered, and purged with argon for 5 minutes to initiate anaerobic conditions. The biosolids containing bottles were then placed in a temperature-controlled room at 37 °C and continuously mixed using a bench top shaker. The samples were given two weeks to equilibrate after which each sample received 1 mL cellulose solution (100 gL⁻¹). The experimental setup included three nanomaterial treatments and a positive control containing Ag⁺ (AgNO₃ was used as the source of Ag⁺). Four dosing levels were examined (0.5, 1, 5 and 100 mgL⁻¹ as Ag). For consistency, after dosing with the various silver concentrations, de-aired DI-water was added to each serum bottle to achieve a final volume of 150 mL. A negative control composed of biosolids only (no addition of Ag) was also examined. All samples were prepared in triplicates.

2.3 Sampling and Analysis

2.3.1 Biogas Analysis

Gas sampling occurred immediately after the addition of treatments (time 0) and after 2, 5, 10, 17 and 28 days. The duration of the experiment was chosen to mimic the anaerobic biosolids retention time (25-30 days) at the WWTP from where the sludge was collected. The gas volume generated was measured using an 18 gauge needle and an airtight glass syringe according to US EPA OPPTS method 835.3400 (23). The gas composition (O₂, CH₄, and CO₂) was evaluated

using a gas chromatograph equipped with a thermal conductivity detector (GC/TCD; Agilent 6980N).

2.3.2 Taxonomical Analysis

Concurrent to the gas analysis, a 2 ml sludge aliquot was taken from each serum bottle and centrifuged at 5000 rpm for 15 minutes. After decanting the supernatant, the sample was preserved at -80 °C for taxonomical analysis. Total genomic DNA was extracted from the anaerobic sludge samples using a QIAamp stool DNA mini kit as described by Dowd et al. (24). Because of the intrinsic uncertainty of the analysis, the data are presented to demonstrate a general trend of the microbial communities and not for their absolute values.

2.3.3 Silver Speciation Analysis by X-ray Absorption Spectroscopy (XAS)

To evaluate changes in Ag speciation, X-ray absorption spectroscopy (XAS) was conducted at Sector 10-ID (25) of the Advanced Photon Source at Argonne National Laboratory (ANL), Argonne, IL. The analysis followed the experimental setup and sample analysis described by Lombi et al. (26). To avoid changes in metal speciation during the sample preparation process, the anaerobic biosolids were dosed with the particular AgNP treatment in its natural liquid state, under anaerobic conditions, and immediately analyzed under a real-time setting in a reaction cell to observe the reactions occurring. In a 1 mL cell, 800 µL of anaerobic sludge was added. Before analysis, 200 µL of the BPEI-AgNPs (500 mgL⁻¹) was injected into the cell giving a final concentration of 100 mgL⁻¹ silver and a known concentration of sulfide. The contents of the cell were examined by XAS utilizing a quick scan set up. In addition to the real-time analysis of the BPEI-AgNPs in natural sludge, additional samples with elevated sulfide levels in the sludge were analyzed operating under the exact experimental settings as described above.

2.3 Modelling Speciation of Dissolved Ag (MINTEQ)

The chemical speciation of dissolved silver (Ag^+) in anaerobic biosolids is an important factor for understanding the toxicity, mobility and bioavailability of the metal within the environment (27). The chemical speciation of silver was modeled using Visual MINTEQ, an equilibrium model that was developed based on MINTEQ2 (28). The model generates data used to determine the chemical speciation and bioavailability of dissolved Ag^+ after introduction to the anaerobic biosolids. The visual MINTEQ2 input parameters, used for the study, were based on the initial characteristics of the utilized anaerobic biosolids (Table S1 in SI). These parameters include pH, redox potential, and dissolved metals. Sulfate, sulfide, phosphate, chloride, and nitrate concentrations were also included in the model due to their importance in dissolved metal complexations (29).

2.4 Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) test (SigmaPlot 12.0, Systat Software Inc.) where the statistical significance was calculated using untreated samples (control) and treated samples (AgNO_3 and AgNPs) and a p -value > 0.05 were considered as significant.

3.0 Results and Discussion

3.1 AgNPs Characterization

Silver nanoparticles with three different capping agents were characterized. The fundamental characteristics that may contribute to the toxicological behavior of these AgNPs (e.g., the (HDD) and the (ζ)) are summarized in Table S2 of the SI (30). The AgNPs synthesized for this experiment were spherical and in the size range between 10 and 15 nm, well below the size at which nanoparticles exhibit unique properties (31). The AgNPs size distributions obtained from

the TEM images (Figures S1, S2, and S3, SI) were consistent with those obtained by dynamic light scattering (DLS). The particles used varied in surface charge which ranged from -40 to +40 mV and stabilization mechanisms (see Tables S2, and SI). It is noteworthy to point out that the Ag^+ concentrations in the AgNP suspensions were minimal since an ultrafiltration system was used to clean the suspensions (32).

3.2 Biogas Volume

The cumulative biogas production after the 28 days anaerobic incubation period is shown in Figure 1. At relatively low doses ($< 5 \text{ mgL}^{-1}$ as Ag), there was no statistically significant difference ($p>0.05$) between the treatment types. These results are in line with previous studies that found methanogenesis to be unaffected by AgNPs at similar concentrations (33, 34). Interestingly, as the AgNPs concentrations increased to 100 mgL^{-1} statistically significant differences regarding inhibition were observed between treatment types. At that level, the biogas production of serum bottles containing citrate and PVP coated AgNPs showed no significant difference ($p>0.05$) compared to the background (negative control). In the case of citrate-AgNPs, the lack of toxicity associated with these nanoparticles might be caused by aggregation and subsequent settling and as a result, the nanoparticles were less likely to interact with the microbial community. Electrostatically stabilized nanoparticles, like citrate-AgNPs, readily aggregate in solutions with high ionic strength. On the other hand, sterically stabilized nanoparticles like PVP-AgNPs are more stable in solutions with high ionic strength like sludge (35). The lack of surface charge on PVP-AgNPs may have resulted in lower attraction between the nanoparticles and the negatively charged bacterial membrane thus manifesting a low toxicity. While citrate and PVP-AgNPs showed no toxicity at the 100 mgL^{-1} level, both the Ag^+ and the BPEI-AgNPs treatments showed near complete inhibition of the anaerobic biogas production

process. The oxidative dissolution of Ag^+ may play a role in the aerobic toxicity of AgNPs, but the anaerobic conditions are unfavorable for such dissolution (36). Since all of the synthesized AgNPs were in the same size range, size as a parameter of toxicity, can be ruled out leaving surface characteristics as the distinguishing factor among the particles. Citrate-AgNPs have a negative surface charge, PVP-AgNPs are neutral, and BPEI-AgNPs are positively charged. Taking into account that the vast majority of the bacterial cells in the anaerobic sludge are negatively charged (37), it is probable that the surface charge of the BPEI-AgNPs would give these particles affinity to the negatively charged bacterial walls while the other particles would not behave in the same manner.

These findings were not surprising as similar toxicity trends were observed with these nanoparticles under aerobic environments (38). Furthermore, Ivask et al. concluded that under aerobic conditions, BPEI-AgNPs impact on *E. coli* is specific to this type of nanoparticles (39). The positively charged AgNPs are theorized to attach onto the surface of the bacteria and cause cell lysis through physical interaction.

3.3. Taxonomical Analysis

The biogas data show that at the 100 mgL^{-1} Ag dose level, there was no biogas generation in both the BPEI-AgNPs and the Ag^+ treatments which was supported by the taxonomical analysis conducted on these samples. At 5 mgL^{-1} dose, the biogas production of the BPEI-AgNPs treated samples showed similar trends ($p > 0.05$) to both the negative and positive controls. However, the taxonomical analysis suggests otherwise. For example, Figure 2 represents the taxonomical data for one of the most abundant bacterium present in the samples (Flavobacterium). As can be observed, there seems to be time dependent variability in the response of the bacterium to BPEI-AgNPs as opposed to the negative or the positive control. In this case, it appears that the

1 population of this bacterium is increasing over time in the BPEI treatment and remaining rather
2 constant in the other treatments. Other bacteria evaluated (Table S3, SI) showed similar time-
3 dependent variations.

4 These results seem to reinforce the concept that the functional redundancy often built into a
5 complex bacterial community, like the one found in biosolids, maintains the best level of
6 performance especially at low AgNPs dosing levels (19). The results further support the
7 observation that BPEI-AgNPs toxicity pathways are different than those associated with Ag⁺
8 only (39). It is also important to note that the data shows that dominant bacterial communities
9 responsible for anaerobic degradation are bacteria with a negative surface charge. This is of
10 importance as the only nanoparticles that demonstrated a toxic and inhibitory effect on the
11 digestion process were BPEI-AgNPs which have a positive surface charge.

12 **3.4. XAS**

13 Previous studies indicate that transformation of AgNPs to Ag₂S is to be expected during
14 wastewater transport/treatment. A rapid conversion of AgNPs to Ag₂S during sewage treatment
15 was reported for one AgNP material by Kaegi et al. (40). The mechanism of this process has
16 been studied in detail by Liu et al. who suggested that at high sulfide concentrations, like those
17 present in the anaerobic digesters, oxysulfidation occurs by a direct particle-fluid reaction rather
18 than through an oxidative dissolution of the AgNPs (41).

19 Since the only toxicity observed was with one of the capping agents (BPEI-AgNPs), these
20 particles were chosen for further investigation. To further analyze the impact of sulfide
21 concentrations on the rate of sulfidation, anaerobic biosolids containing two levels of sulfide
22 were investigated; natural sulfide levels in biosolids ~15 mgL⁻¹ and elevated to 100 mgL⁻¹
23 sulfides. The concentration of AgNPs was kept constant at 100 mgL⁻¹.

The XAS spectra demonstrated immediate sulfidation of the nanoparticles upon introduction to the media regardless of the sulfide level (Figure 3). Nevertheless, a higher percentage of sulfidation was observed for the particles in the elevated sulfide sludge. This is of much importance as the rate of sulfidation may have a direct correlation with the toxicity of AgNPs. These results demonstrate that although AgNPs partially sulfidize upon entering the anaerobic sludge media (Table 1), the transformation to Ag₂S was incomplete. The analysis shows that sulfidation kinetics of BPEI-AgNPs are dependent on the concentration of sulfur within the media. As demonstrated, the BPEI-AgNPs in biosolids with natural sulfide levels showed a slower sulfidation rate within the first 24 hours as compared to the same particles in biosolids with elevated sulfide levels. Therefore, based on the amount of sulfide in the system, the sulfidation kinetics vary allowing BPEI-AgNPs to retain their metallic form for various times. Hence, allowing the particles to exhibit their particle specific toxicity.

3.5 Speciation of Dissolved Metals in Anaerobic Sludge (MINTEQ)

The chemical speciation of dissolved Ag⁺ in anaerobic biosolids was modeled using the biosolids parameters as input values summarized in Table S1, SI. The input values produced chemical speciation data that were used to determine the form in which the dissolved silver has within the anaerobic biosolids and also the percentage of the dissolved metals present in their ionic state. Due to the nature of the media under investigation, it is not possible to identify and include all possible interactions in the system. Nevertheless, the chemical species that are known to impact the speciation of AgNPs were measured and considered for this simulation.

Based on these results (Table S4, SI), less than 1% of the dissolved Ag present in the system would be free Ag⁺. At sulfide concentrations of ~15 ppm, found naturally in the biosolids, Ag would be predominantly associated with hydrogen sulfide (AgHS_(aq)) (94.61%) and also sulfide

(AgS⁻) (3.427%). A small portion of the Ag was also associated with the chloride as (AgCl_(aq)) (1.13%). These results are similar to those reported by Bolyard et al. (42). The data further exclude the toxicity of BPEI-AgNPs due to oxidative dissolution of Ag and further supports the conclusion that other toxicity pathways are the primary mechanism for the toxicity of BPEI-AgNPs.

4.0 Implications

At low concentrations, AgNPs seem not to drastically impact anaerobic degradation which is the result of the functional redundancy built within the microbial community and not the lack of toxicity of AgNPs. At relatively high concentrations the cationic BPEI-AgNPs demonstrate elevated toxicity as compared with PVP-AgNPs and the anionic citrate-AgNPs. Because of the lack of oxidative dissolution in anaerobic environments, the results further support previous research that suggests a different toxicity mechanism is in place with BPEI-AgNPs as compared to other types of AgNPs. It must be noted that BPEI-AgNPs toxicity did not manifest until relatively high concentrations (100 mgL⁻¹ as Ag). Under normal operating conditions in a WWTP, AgNPs concentrations would not be expected to reach such levels. Thus, it is reasonable to believe that most WWTPs will be able to manage AgNPs without negatively impacting their operations. The results of this study also demonstrate that a one size fits all approach to the evaluation of environmental health and safety of nanoparticles may not necessarily be accurate. Even within one class of nanoparticles, there appear to be differences that drastically impact their behavior in the environment.

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Table 1. Ag speciation as identified by LCF of k-edge XANES spectra. Silver species proportions are presented as percentages. The R-factor indicates the goodness of fit.

Sample	Time (hr)	Ag ₂ S (%)	Ag ₀ (%)	R-factor (x 1000)
100ppm BPEI-AgNPs in Sludge (<i>15 mgL⁻¹ Sulfide</i>)	0	24	76	0.13
	4	53	47	0.13
	24	95	5	0.32
100ppm BPEI-AgNPs in Sludge (<i>100 15 mgL⁻¹ Sulfide</i>)	0	95	5	0.5
	4	97	3	0.33
	24	97	3	0.29

Figure 1. Cumulative gas volume production of anaerobic biosolids after exposure to different concentrations of AgNPs or Ag^+ for a duration of 28 days.

Figure 2. Flavobacterium species among different treatments at 5 mgL⁻¹ of silver

Figure 3. Ag-K α XAS spectra of Ag₂S, Ag metallic as pure phases and BPEI-AgNPs reacted with the anaerobic biosolid material at natural (15 mgL⁻¹) and elevated (100 mgL⁻¹) sulfide levels over a 24 hr reaction period.