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Bacterial uptake of silver nanoparticles in the presence of humic acid and $AgNO_3$

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Abstract−Silver nanoparticles (AgNPs), potent antibiotic materials, have been found to cause cell-membrane damage and produce reactive oxygen species (ROS). The resultant structural change in the cell-membrane could cause an increase in cell permeability of silver ions and AgNPs. To address this issue further, in-vivo and in-vitro cytotoxicity testing of as-made nanomaterials was conducted to quantify and assess their nanotoxicity. Considering the behavior of AgNPs in the environment, toxicity may be reflected by differences in their physicochemical properties (size, agglomeration rate, adsorption properties on humic acid) dependency and toxicity depression. Therefore, we investigated the effect of the cellular uptake of AgNPs with the kinetics of agglomeration and adsorption. The amount of agglomerated and adsorbed AgNPs with sizes of <14 nm was higher than that for AgNPs with sizes of 90 and 140 nm. For 90 and 140 nm sized AgNPs, adsorption was more significant than agglomeration. It is noteworthy that the normal concept that smaller sized AgNPs are taken up more readily may be in error in cases of interactions of abiotic factors.

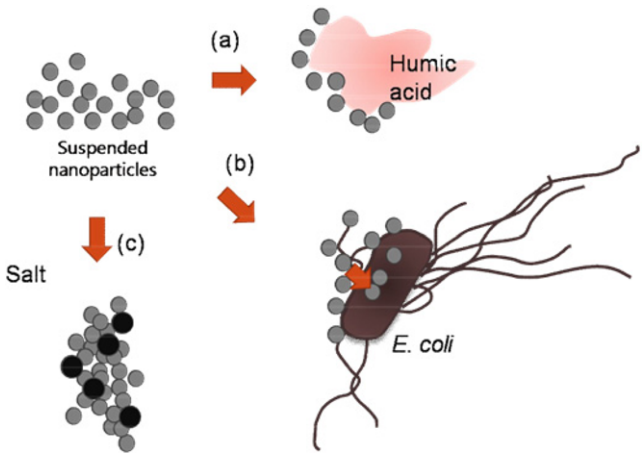
Key words: Silver Nanoparticle, Agglomeration, Adsorption, Humic Acid, Salinity, *Escherichia coli*

INTRODUCTION

Concern regarding engineered nanomaterial safety is currently receiving considerable attention in the scientific, academic, industrial, and regulatory communities because of uncertainties regarding the environmental impact of such materials [1]. Silver nanoparticles (AgNPs), as a water pollutant, have been reported to affect numerous environmental systems, such as bacteria cells [2], aquatic organisms such as zebra fish, and algae [3]. Recently, it has been published that the antibacterial mechanism of AgNPs is related to the formation of free radicals and subsequent free radical-induced membrane damage [4,5]. Hwang et al. reported a synergistic toxic effect of the AgNPs and silver ions [6]. The ions move into the cells and produce reactive oxygen species (ROS). In addition, membrane damage caused by the AgNPs caused an increase in cell permeability, leading to an uncontrolled transport through the cytoplasmic membrane and ultimately cell death [7,8].

However, toxicological investigations of nanomaterials often suffer from problems with stability or interactions with natural matter in the test system, because it is necessary to mimic environment conditions in cases of toxicity tests. The behavior of engineered nanoparticles in the environment is affected by environmental conditions such as the presence of natural organic matter (NOM) and salinity, resulting in agglomeration, deposition, or adsorption [9,10]. The agglomeration of nanoparticles occurs in the early stages of environmental exposure, and this can determine the fate, and limits the transport, of nanoparticles into cells.

Particle size is indirectly the dominant factor in determining the rate of uptake and is related to agglomeration [11,12]. Considering the behavior of AgNPs in the environment, the degree of toxicity may be different, as a function of size dependency and toxicity depression. In addition, the behavior of AgNPs in the environment appears to be related to extrinsic abiotic parameters such as ionic strength (IS) and the presence of humic acids (HA). Herein, humic acid, the main component of NOMs, is capable of absorbing metals, hydrous metal oxides, and chelating multivalent cations, because HA contains a skeleton comprised of alkyl and aromatic units [13]. When HA is adsorbed on the surface of nanoparticles, a pH-dependent charge develops that is influenced by electrostatic interactions, with, for example, mineral particles [14]. At a high pH where nanoparticles are negatively charged or nearly uncharged, the amount of HA absorbed is low. Therefore, interactions between AgNPs and



Scheme 1. Three cases of suspended AgNPs in environment exposure; (a) adsorption with humic acid, (b) cell adsorption and uptake, and (c) agglomeration with salt.

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HA may be influenced by the concentration of HA in the aqueous phase. The existence of branches in HA molecules may result in the formation of looser coils, or network formation may have an effect on the extent of agglomeration with ionic strength. Because HA can have macromolecular characteristics, the absorption of HA to AgNPs can also be compared with ionic strength behavior.

When AgNPs are exposed to the environment, they undergo interactions with environment factors, diffusion in aqueous phases, adsorption to cell surfaces, and uptake into the cell (Scheme 1). The focus of this study is on preferred behavior and a comparison of this behavior with toxicity. Assuming the dominant behavior of AgNPs involves interactions with salts and humic acid, the amount of AgNPs taken up or their adsorption on cells is related to the sum of the free AgNPs in suspension minus the amount that interacts with IS and HA. *Escherichia coli* (*E. coli*), which has the ability to grow in harsh environments, was used as the conceptual model for examining AgNPs exposure.

EXPERIMENTAL

1. Sample Preparation

Silver powder (Sigma-Aldrich, 99%, <150 nm dia.) was suspended in THF, subjected to ultrasonication (Jinwoo-Alex, JAC 1505), followed by stirring at approximately 400 rpm for 1 day. The THF was allowed to evaporate during the period of stirring (700 rpm). Deionized (DI) water was added to replace the evaporated THF. The mixture was stirred for a further 1-2 days until the THF had completely evaporated. The total Ag concentration was measured by inductively coupled plasma spectrometry (ICP; Shimadzu, ICPS 7500). The Ag^+ ion concentration was determined using an ion-selective electrode (ISE; Orion 960) and most of the Ag^+ ions (>90%) were removed by a potentiostatic method with a three electrode-electrochemical cell (pseudo-reference electrode, silver wire; working electrode, platinum plate). The size distribution of the AgNPs was examined by high-resolution transmission electron microscopy (HR-TEM, JEOL, JEM-3010) using image analysis software (SIMAGIS, Smart Imaging Technologies). The average hydrodynamic diameter was calculated with a number-based distribution. We suggest that nanofiltration methods be used to control nanoparticle size; membranes prepared from cellulose nitrate (ave. pore size 200 nm), cellulose ester (ave. pore size 100 nm), and polycarbonate (isopore 50 nm). Average hydrodynamic diameters of AgNPs filtered were 14, 90, and 140 nm.

2. Adsorption Test

AgNPs may agglomerate with salt (Ag^+ and $AgNO_3$) in an aqueous phase and the agglomeration rate was measured by a dynamic light scattering (DLS, Otsuka, ELS-8000) method. This test was conducted at room temperature using a multi-stirrer (100 rpm). HA (10, 50, and 100 mg/L) was mixed with an aqueous solution containing AgNPs. The pH was adjusted to a value of 6.0. The mixture was stirred for 2 hr and samples were taken periodically, filtered using nanofiltration method (50 nm isopore, Millipore). Total silver concentration of solution containing AgNP and Ag^+ was measured by ICP, and silver ion was easily measured by pAg meter. Therefore, the concentration of AgNP without Ag^+ was the difference between concentration obtained by ICP and that measured by pAg meter.

3. Bacterial Cytotoxicity

E. coli (ATCC8739) was used for the cytotoxicity test. *E. coli* was inoculated in 40 mL of Luria-Bertani (LB) and nutrient broth medium, respectively, and incubated at 37 °C for 18 h. The cells were centrifuged at 1,000 ×g for 10 min and washed 3 times with 40 mL of 150 mM phosphate-buffered saline (PBS, pH 7.2). The bacterial suspension was diluted and an initial population of 10^6 CFU/mL was resuspended in 28 mL of 10 mM phosphate buffer (pH 7.1). Then, 1 mL of AgNPs suspension was added to the indicated final concentrations. To establish anaerobic conditions, the solution was sparged with N_2 gas for 10 min before adding silver ions and throughout the silver-ion treatment. The solutions were exposed to silver ions (acted as salts) for predetermined durations with magnetic stirring. The number of viable cells (N and N_o) was counted using the plate-count method. The test bath contained 0.3 mL of buffer, 27.7 mL of DI water, 1 mL of microbe sample, and 1 mL of AgNP sample.

RESULTS AND DISCUSSION

1. Agglomeration of AgNPs in Environment

Agglomeration properties have been reported [15-17]; however, they are not well understood, particularly with respect to how many nanoparticles are agglomerated, how particles become bigger, or how fast they agglomerate. Therefore, herein, before adding the bacteria in the AgNPs solution, we tested interaction between abiotic factors (HA and IS) and AgNPs. Agglomeration feature was correlated with IS values (Fig. 1), while adsorption feature was correlated with HA (Fig. 2).

AgNPs underwent agglomeration depending on the ionic strength of the solution (Fig. 1). The agglomeration rate constant, defined as the slope of the initial agglomerate size versus time, increased with ionic strength (<0.1 μM) (Fig. 1(a1)). At a high ionic strength (>0.1 μM), the AgNPs agglomerates increased in size, but did not increase with a further increase in ionic strength (Fig. 1(a2)). This can be referred to as the pseudo-steady state for suspension stability by

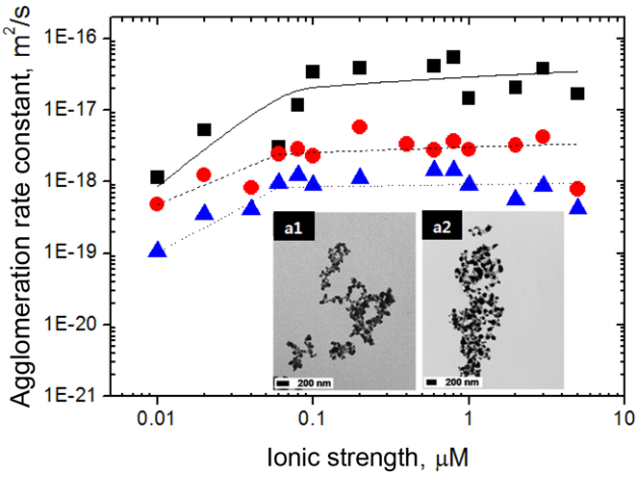

Fig. 1. Agglomeration rate constant of AgNPs with ionic strength; 14 nm (■), 90 nm (●), and 140 nm (▲) of average diameter of AgNPs (Inset: TEM images of AgNPs-salt controlled with (a1) 0.1 μM IS, and (a2) 10 μM IS).

interactions between AgNPs and salts. The agglomeration of the AgNPs accelerated with increasing ionic strength. AgNPs of <20 nm average size were so unstable that they readily agglomerated in the presence of salt, even though they agglomerated with humic acid. For 14 nm sized AgNPs, the results varied (agglomeration rate constant) by ±25% change of variables which showed that agglomeration with IS varied between 10.54 and −17.69%, but the agglomeration with HA was 8.13% and −13.69%. AgNPs agglomeration induced the precipitation of AgNPs. Under this perspective, the current findings for diffusion-limited agglomeration and size-dependent mean mass uptake determined for AgNPs may be also valid for most nanoparticles.

2. Adsorption of AgNPs in the Environment

Interactions with HA may be not based on agglomeration but, rather, adsorption. Some AgNPs were coated by HA, resulting in a faint black color in TEM images (Fig. 2), which formed a network in cases of small amounts of HA. Depending on the HA concentration, AgNPs coated with HA were networked with each other (Fig. 2(b1)) and adsorbed by HA aggregates or clusters (Fig. 2(b2)). Enhanced aggregation can occur through charge neutralization and bridging mechanisms caused by fibrillar attachment. HA particles aggregated in an aqueous phase and the aggregate size was in the range from 68 nm to 320 nm for 0.25%- 1% HA. Each concentration of AgNPs and HA was correlated and the results are shown in Fig. 2. Importantly, both of them are dependent on the size of AgNPs, because the smaller the size, the bigger the surface area. The adsorption of AgNPs to humic acid can be considered as pseudo-second order reaction involving a multi-component system [18].

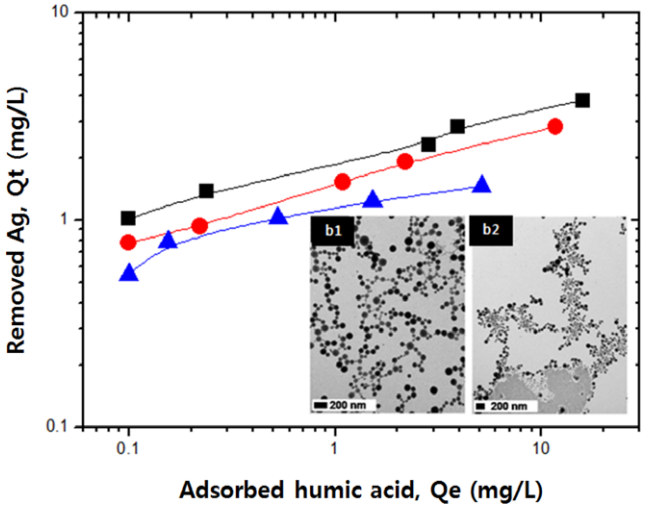

Fig. 2. Adsorption rate of AgNPs on humic acid; 14 nm (■), 90 nm (●), and 140 nm (▲) of average diameter of AgNPs. (Inset: TEM images of AgNPs-humic acid controlled with (b1) 10 mg/L HA, and (b2) 100 mg/L HA).

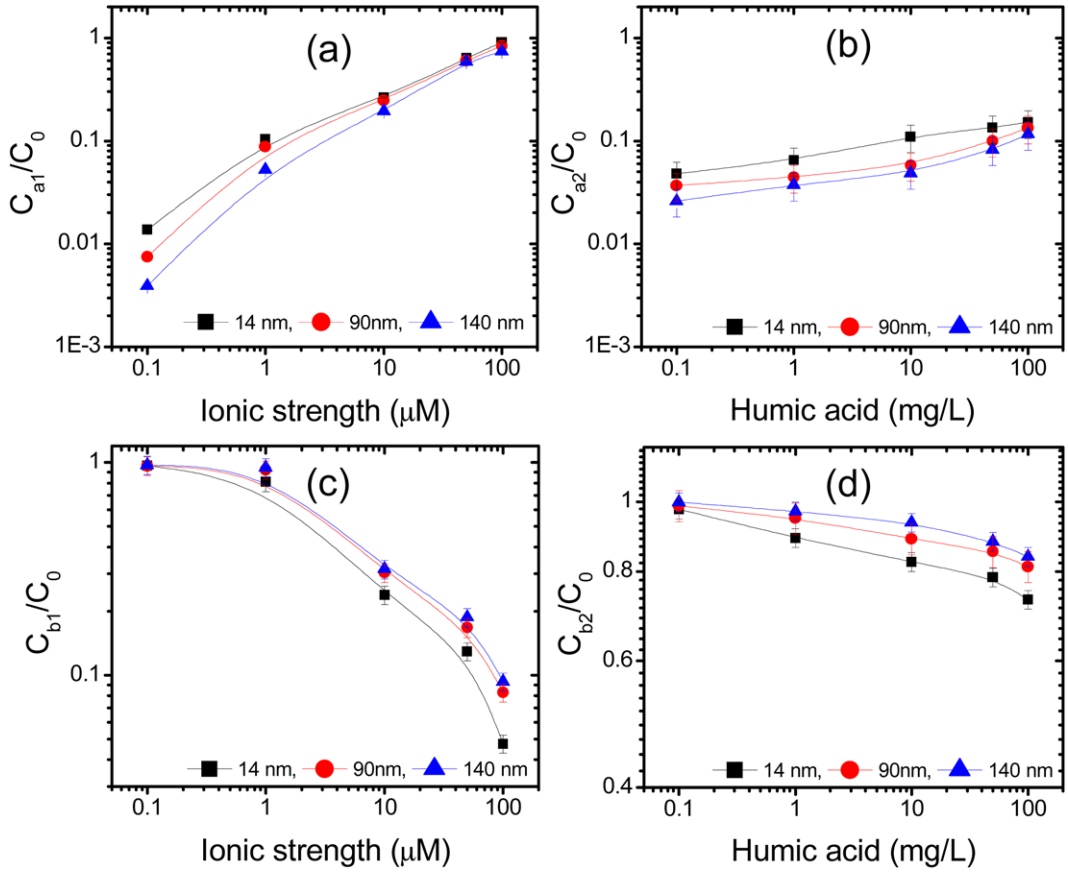

Fig. 3. Agglomeration and free suspension of AgNPs; (a) agglomerated AgNPs (a1 in Fig. 1) with ionic strength; (b) adsorbed AgNPs (a2 in Fig. 1) with humic acid; (c) free suspended AgNPs (b1 in Fig. 2) with ionic strength and *E. coli*; (d) free suspended AgNPs (b2 in Fig. 2) with humic acid and *E. coli*.

$t/Q_t=[1/2\cdot k\cdot Q_e^2]+t/Q_e$

Where, k is the rate constant for adsorption (g/mgûmin), Q_e is the amount of metal adsorbed at equilibrium (mg/g), and Q_t is the amount of metal adsorbed at any time, t (mg/g). Specific adsorption reached the equilibrium state within 30 min independency on humic acid concentration. The amount of adsorbed AgNPs was increased inversely with the size of the AgNPs. The rate constants for AgNPs adsorption were increased inversely with the size of AgNPs, because of the dependency on diffusion rate.

In the case of environment exposure, interactions with HA were less important than that of ionic strength. The amount of AgNPs removed by ionic strength and HA is shown in Fig. 3. In the measurement range (0.1-100 μM) for ionic strength, almost all of the AgNPs were removed (Fig. 3(a)). In addition, the amount of free suspended AgNPs decreased with ionic strength and a ratio of free AgNPs to initial AgNPs (C_{b1}/C_0) was below 0.1 at >10 μM ionic strength. The difference in the removal AgNPs (C_{a1}/C_0) and free AgNPs (C_{b1}/C_0) is related to the amount of adsorbed and uptake of AgNPs in a cell. Less than 1% of the initial AgNPs concentration could affect *E. coli* cells. Otherwise, the effect of HA on AgNPs was less than that for ionic strength (Fig. 3(b)). The amount of AgNPs removed by adsorption to HA was below 10% in the measurement range tested (Fig. 3(b)). In addition, most AgNPs (<80%) were suspended (Fig. 3(d)). Interestingly, AgNPs in the environment can be removed by IS, rather than HA. This finding is supported by the cytotoxicity tests using *E. coli*.

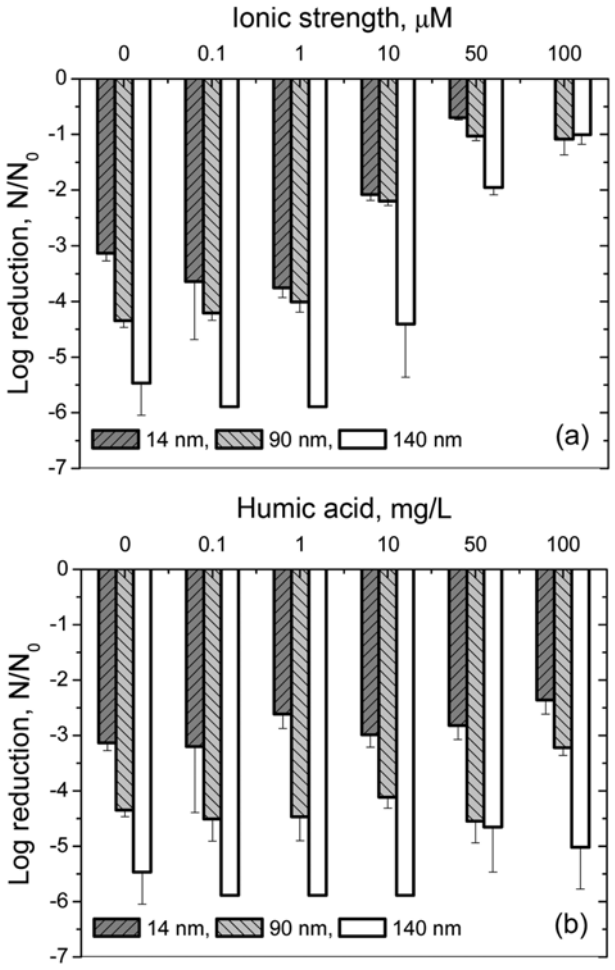

Fig. 4. Inactivation of *E. coli* by AgNPs agglomerate depending on (a) HA and (b) IS; (a) 0.4 mg/L AgNPs; (b) 0.8 mg/L AgNPs. Where, N is number of viable *E. coli*.

3. Cell Uptake of AgNPs

The inactivation of *E. coli* was affected by the agglomeration and adsorption of AgNPs (Fig. 4). Inactivation was significantly more dependent on agglomeration. The agglomeration of AgNPs decreased by adding salt and the inactivation of *E. coli* was decreased. Otherwise, adsorption was merely sensitive to the concentration of humic acid rather than ionic strength. Importantly, the amount of cellular uptake was decreased with decreasing size of AgNPs, and the inactivation of *E. coli* was decreased with decreasing size of AgNPs. The shape of the whole bacteria and the viability of the cytoplasm were not obviously modified by AgNPs. On the contrary, the external region of the bacteria clearly displays an outer shell with a high electronic density corresponding to a layer of AgNPs

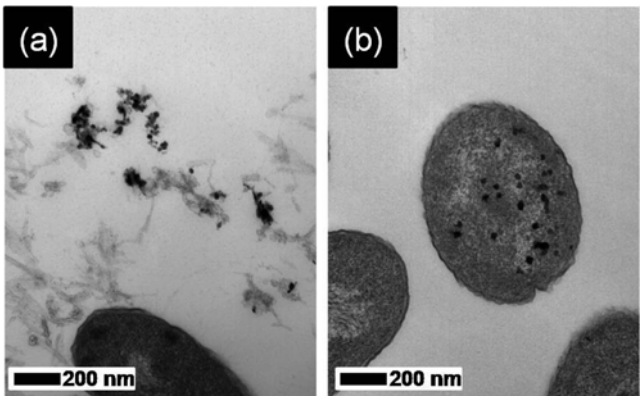

Fig. 5. The morphological change of *E. coli* after exposure of AgNPs suspension containing (a) the humic acid and (b) salt.

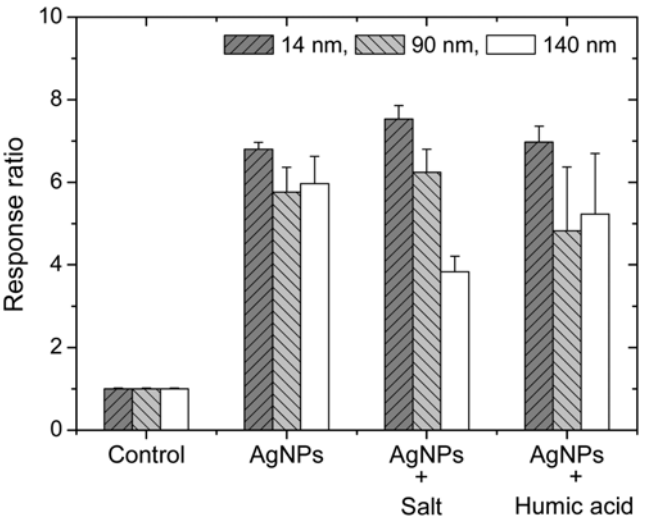

Fig. 6. Oxidative stress induction (*soxS* gene expression) by AgNPs agglomeratedepending on humic acid and salt. AgNPs samples had average diameter of 14 nm (size 1), 90 nm (size 2), and 140 nm (size 3).

adsorbed to the outer membrane of the cells. Small surface charges allowed primary particles to overcome the electrostatic repulsions, and nanoparticles rapidly agglomerated. The cellular uptake AgNPs interfered with the agglomerate of salt or adsorption to HA (Fig. 5).

Even though AgNPs agglomerated in the solution and adsorbed, the cells might uptake AgNPs and experience the oxidative stress (Fig. 6). Intercellular oxidative stress (*sox*S gene expression) was dependent on the size of AgNPs and increased with the decrease in the size of AgNPs. Oxidative stress was related with penetration efficiency of AgNPs into the cell [19]. Smaller size of AgNPs is more favorable to cell uptake. However, oxidative stress didn't have effects by the presence of $AgNO_3$ and humic acid.

CONCLUSIONS

Silver nanoparticles (AgNPs) were exposed to *E. coli* in an aqueous solution containing humic acid and salts. The experimental results are discussed in relation to the adsorption behavior of humic acids and the cellular uptake of AgNPs. The inactivation of *E. coli* was depressed through the specific processes of agglomeration and adsorption. The agglomeration of AgNPs decreased by adding salt, and the inactivation of *E. coli* was decreased. Importantly, the amount of cellular uptake was decreased with the decreases in the size of AgNPs, and the inactivation of *E. coli* was decreased as well. The morphology of *E. coli* cells changed with AgNPs uptake, and cytoplasm aggregation was observed. With the assumption that the behavior of AgNPs is predominantly due to interactions with salt and humic acid, we conclude that the amount of AgNPs uptake or adsorption to cells is related to the sum of free AgNPs in suspension minus the amount that interacts with IS and HA.

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