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Binding of Silver Nanoparticles to Bacterial Proteins Depends on Surface Modifications and Inhibits Enzymatic Activity

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Here we describe results from a proteomic study of protein−nanoparticle interactions to further the understanding of the ecotoxicological impact of silver nanoparticles (AgNPs) in the environment. We identified a number of proteins from *Escherichia coli* that bind specifically to bare or carbonate-coated AgNPs. Of these proteins, tryptophanase (TNase) was observed to have an especially high affinity for both surface modifications despite its low abundance in *E. coli*. Purified TNase loses enzymatic activity upon associating with AgNPs, suggesting that the active site may be in the vicinity of the binding site(s). TNase fragments with high affinities for both types of AgNPs were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Differences in peptide abundance/presence in mass spectra for the two types of AgNPs suggest preferential binding of some protein fragments based on surface coating. One high-binding protein fragment contained a residue (Arg103) that is part of the active site. Ag adducts were identified for some fragments and found to be characteristic of strong binding to AgNPs rather than association of the fragments with ionic silver. These results suggest a probable mechanism for adhesion of proteins to the most commonly used commercial nanoparticles and highlight the potential effect of nanoparticle surface coating on bioavailability.

Introduction

Synthetic nanoparticles, defined by their size (≤100 nm), are used in a large array of industrial applications that are dependent on nanotechnology, ranging from high-end electronics to common consumer products. The sheer number of applications for these new materials now (e.g., over 800 consumer products in 2009 (*1*)) and in the future as the industry continues to grow will lead to the production of several tons of nanoparticles in the coming decade (*2*). The risks associated with the possible release of synthetic nanoparticles to the environment are unknown; however, some studies have already documented the measurable release of nanoparticles into the environment (*3, 4*).

Silver nanoparticles (AgNPs) are one of the few types of synthetic nanoparticles to receive considerable attention for their possible environmental impact. Not only is their composition a potential concern—the silver ion (Ag^+) is a known toxicant—but their high effectiveness as a wide spectrum biocide and their growing use in consumer products including food containers, socks, towels, and household appliances makes them most likely to be released into the environment in the greatest quantities (*5*). Although at certain levels AgNPs can be toxic to all organisms, a significant environmental impact of the release of AgNPs may be on microbial communities. Release of AgNPs from consumer products or production facilities holds the potential to influence bacteria in downstream wastewater treatment plants (*6*) as well as bacterial communities in soil or natural waters (*7, 8*). A detailed understanding of the mechanisms of toxicity, environmental transport, and bioavailability of AgNPs will help regulators assess the environmental risk associated with AgNPs and may have significant implications for predicting the toxicity of a wide range of other related nanomaterials.

Recent efforts to describe the toxicity of AgNPs toward microorganisms have revealed a potentially important role for biomolecules in AgNP toxicity and bioavailability. Because AgNPs have been shown in some cases to be more toxic to bacteria than free Ag^+ ions (*9–12*), the toxicity mechanism may be controlled by the nanoparticle surface, as is the case for other nanomaterials (*13*). This hypothesis has been corroborated by the observations that factors influencing nanoparticle surface reactivity, including size (*9, 14*) and shape (*15*), influence AgNP toxicity. One proposed mechanism to explain how the AgNP surface can be seemingly more toxic than free Ag^+ ions is that large amounts of surface-associated Ag^0 are oxidized after attachment of AgNPs to biomolecules on the cell, which induces AgNP dissolution (*12, 14*). Because of the targeted delivery of Ag^+ ions in close proximity to, and possibly inside, the cell, the bulk silver concentration needed to exceed the lethal limit is significantly lower in the case of AgNPs (*11*). On the basis of these studies, AgNPs may be more toxic than an equal mass of aqueous Ag^+ due to dilution/diffusion effects. It has also been suggested that AgNP toxicity stems from a physical process whereby they disrupt the cell membrane and/or penetrate inside the cell (*16, 17*). Proteomic studies demonstrate that there is a distinct cellular response to the presence of AgNPs including the production of additional cell envelope protein precursors, suggesting destabilization of the outer membrane and collapse of the proton motive force (*11, 14*). Despite a lack of general consensus on the mechanism of toxicity of AgNPs to bacteria, the latter two processes, both surface-controlled mechanisms, are dependent on the direct contact of AgNPs with biomolecules. It seems likely then that certain proteins (or sites on a protein surface) may have increased reactivity or affinity for AgNPs.

In this report, we used a proteomics approach (*18*) to (1) identify *Escherichia coli* proteins with a high affinity for AgNPs, (2) characterize binding between a purified bacterial protein, tryptophanase (TNase), identified from the soluble proteome of *E. coli*, and test the effect of binding on enzymatic

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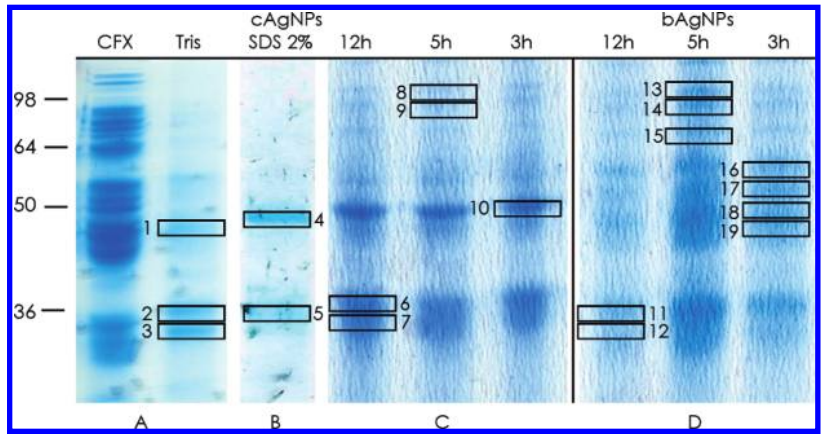

FIGURE 1. SDS-PAGE gel of (A) *E. coli* CFX and proteins from CFX that remain bound to cAgNPs after washing with Tris, (B) proteins that remain associated with cAgNPs after washing with 2% SDS, (C) *E. coli* CFX proteins associated with cAgNps as a function of incubation time, and (D) *E. coli* CFX proteins associated with bAgNPs as a function of incubation time. The boxes indicate bands that were excised and analyzed by LC-MS/MS.

activity, and (3) identify regions of the purified protein where AgNP binding occurs and characterize the effect of surface modifications on the binding.

Experimental Section

Materials. Commercial AgNPs with two surface modifications were purchased from Nanosys GmbH (Wolfhaden, Switzerland): bare (bAgNPs) and carbonate coated (cAgNPs). Previous studies (*12*) combined with dynamic light scattering size distribution, zeta-potential measurements, and transmission electron microscopy (TEM) images indicate relatively stable AgNP solutions with similar morphologies and broad size distributions (average particle size $\approx$ 30 nm) (Figures S1−S3, Supporting Information). All other chemicals were purchased from Sigma-Alrich unless otherwise noted. AdhP from *E. coli* was overexpressed and purified according to standard protocols. Purified *E. coli* TNase used in this study was generously provided by Prof. Robert Phillips (University of Georgia).

Protein Identification. To identify proteins with high binding affinities to AgNPs, 10 μg/mL (as determined using the Bradford assay) of soluble proteins from the cell free extract (CFX) of *E. coli* was reacted overnight with 100 ppm of AgNPs (SI-1). This concentration of AgNPs diluted in deionized water prevented significant dilution-induced aggregation [minor aggregation was observed for bAgNPs (Figure S1, Supporting Information)] and provided a suitable amount of nanoparticle−protein conjugates for collection. Proteins associated with the AgNP pellet following centrifugation (SI-2, Supporting Information) were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) by directly loading the AgNP/protein pellet into gel-loading wells.

Whole-Protein Binding Experiments. To verify the binding of single proteins to AgNPs, aggregation assays were performed using a Shimadzu spectrophotometer. In this assay, increasing quantities of adsorbed proteins prevent AgNP aggregation (*19*). Several concentrations of proteins (1−20 μg/mL) were reacted with 10 ppm of AgNPs, and the absorbance was monitored from 300 to 700 nm. To induce aggregation, 1 M NaCl was added to each sample and reacted for 20 min before measuring absorbance (*19*).

Binding of Protein Fragments to AgNPs. Protein fragments were produced by digesting native proteins in chymotrypsin or trypsin overnight at 37 °C in 10 μM $CaCl_2$ and 20 mM Tris-HCl pH 7.7. These fragments were then reacted with AgNPs overnight following the protocol outlined above for full proteins. TNase results were compared to two model proteins (cytochrome *c*, Cytc; bovine serum albumin, BSA) and another *E. coli* protein previously identified by our group for its high affinity to Se nanoparticles (alcohol dehydrogenase, propanol-preferring; AdhP). For reactions with cysteine present as a Ag^+ complexing agent, 10 μM cysteine (predicted equimolar concentration of Ag^+; ~1% of total Ag is present as aqueous Ag^+ (*12*)) was prepared from a fresh stock solution and kept on ice before the reaction. Silver nitrate ($AgNO_3$) reactions with protein fragments were performed as above except AgNPs were replaced by 10 mM $AgNO_3$. Excess $AgNO_3$ was removed from solution before analysis by washing the peptides several times using high-affinity C_{18} resin columns (Millipore, Inc.).

The analysis of peptide binding experiments was carried out by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (SI-2, Supporting Information).

Enzymatic Assay. The activity of TNase was quantified before and after binding to both types of AgNPs using the standard TNase activity assay (*20*) (SI-3, Supporting Information).

Results and Discussion

Identification of High-Binding Bacterial Proteins. We carried out SDS-PAGE on *E. coli* CFX reacted overnight with AgNPs to identify proteins that remained associated with the nanoparticles in a competitive binding environment where high-abundance proteins compete with high-affinity proteins. This method has previously been shown to be a good screening technique because AgNPs do not affect protein migration in gels (*21*). Centrifugation of protein−nanoparticle conjugates as a concentration and separation step selectively enhances high-affinity proteins over more abundant, low-affinity proteins (*13, 22, 23*). Figure 1 shows the protein bands corresponding to proteins remaining associated with AgNPs following successive washing steps or after incubation for different lengths of time. Additional gels showing the effect of different pH values, incubation times, and wash steps are shown in Figure S4, Supporting Information. The persistence of only a few dominant bands despite centrifugation and successive washes of increasingly concentrated SDS suggests only a small proportion of the total CFX binds tightly to the nanoparticles (Figure 1A and 1B). Reaction time and pH had negligible effects on the binding of these major proteins (Figure 1C and 1D and Supporting Information Figure S4B and S4C).

The proteins identified do not simply reflect the most abundant proteins from the *E. coli* cytosolic proteome (*24, 25*). This suggests that association of these proteins with AgNPs, particularly those expected to be in low abundance in the

cytosol, is due to their high affinity for and strong binding to the NPs (Table S1, Supporting Information). Interestingly, many (~65%) of the proteins identified are enzymes. The nonenzyme proteins identified were membrane porins, chaperones, or periplasmic peptide-binding proteins (Tables S1 and S2, Supporting Information).

There were significant differences in the identity of bound proteins based on AgNP surface modifications (coated vs bare). Most notably, bands 2, 3, 6, 7, 11, and 12 are located in the similar size vicinity (Figure 1). Yet, bands 11 and 12, which correspond to bAgNPs, show weak or no evidence of the presence of several enzymes present in the corresponding bands for cAgNPs (Table S1, Supporting Information, e.g., cysteine synthase or protein CsiD). Additionally, band 1 (cAgNPs) contains adenylosuccinate synthetase, whereas the corresponding band for bAgNPs (band 19) shows no evidence for that enzyme. These differences point to the importance of AgNP surface modifications in determining the identity of proteins that associate with the nanoparticles.

Despite these observed differences, there was also overlap in protein binding based on surface modifications (Table S1, Supporting Information). A similar overlap in protein binding regardless of surface modification was observed for polystyrene nanoparticles with various surface modifications exposed to protein-rich human plasma (*13*). In that study, surface charge was shown to only influence the strength of protein adsorption but did not cause differences in the identities of major bound proteins (*26*). In contrast, in our system, the surface structure appears to have an effect on but not complete control over the identity of the high-binding proteins from *E. coli*.

Interaction of TNase with AgNPs. Table S1, Supporting Information, identifies tryptophanase (TNase) as a major protein associated with cAgNPs after a 2% SDS wash as well as with bAgNPs, a testament to its strong binding to AgNPs. We also identified outer-membrane protein A and C (OmpA and OmpC) in all the excised bands that corresponded to the correct protein size. Thus, the latter two proteins are also reliably associated with the AgNPs.

We selected TNase for further study due to its high abundance in several gels for both types of AgNPs and its physiological role in attachment to solid substrates in biofilms (*27, 28*). The ability of purified TNase to bind well to both types of AgNPs was confirmed by aggregation assays. Low quantities (1 μg/mL) of TNase protect both types of AgNPs from NaCl-induced aggregation (Figure S5, Supporting Information). In the absence of TNase, aggregation occurs rapidly and the spectrum typical of AgNPs disappears due to sedimentation of the aggregates. In contrast, if TNase is present at 1 or 10 μg/mL, aggregation and sedimentation are largely prevented and the AgNPs retain a spectrum (Figure S5, Supporting Information).

Binding to AgNPs also affects the enzymatic activity of TNase (Figure 2). When bound to AgNPs, TNase activity decreased significantly: by 50% for cAgNPs and by over 90% for bAgNPs. These results suggest that both types of AgNPs have an inhibitory effect on the activity of this enzyme; however, the exact mechanism of inhibition is unknown. Considering the relative sizes of TNase (~5 nm) and the AgNPs (~30 nm), the binding of the protein to the AgNPs may alter its conformation, distorting its 3D structure. The inhibitory effect of AgNPs on enzymatic activity demonstrates a potential mechanism for the biocidal effect of AgNPs.

Fingerprinting High-Binding Protein Fragments. In order to understand the mechanism of TNase binding to both AgNPs, we exposed two sets of TNase fragments (created by digesting the purified protein with two different digestion enzymes) to both types of AgNPs and identified the peptides associated with AgNPs using MALDI-TOF MS. Only a small fraction of the total protein fragments generated from

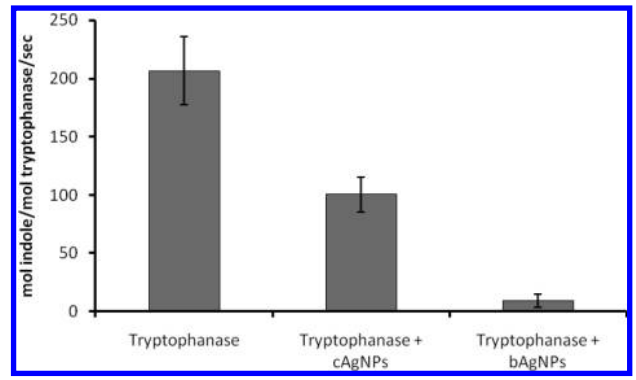

FIGURE 2. Enzymatic activity of TNase in the absence of AgNPs and after incubation with cAgNPs and bAgNPs.

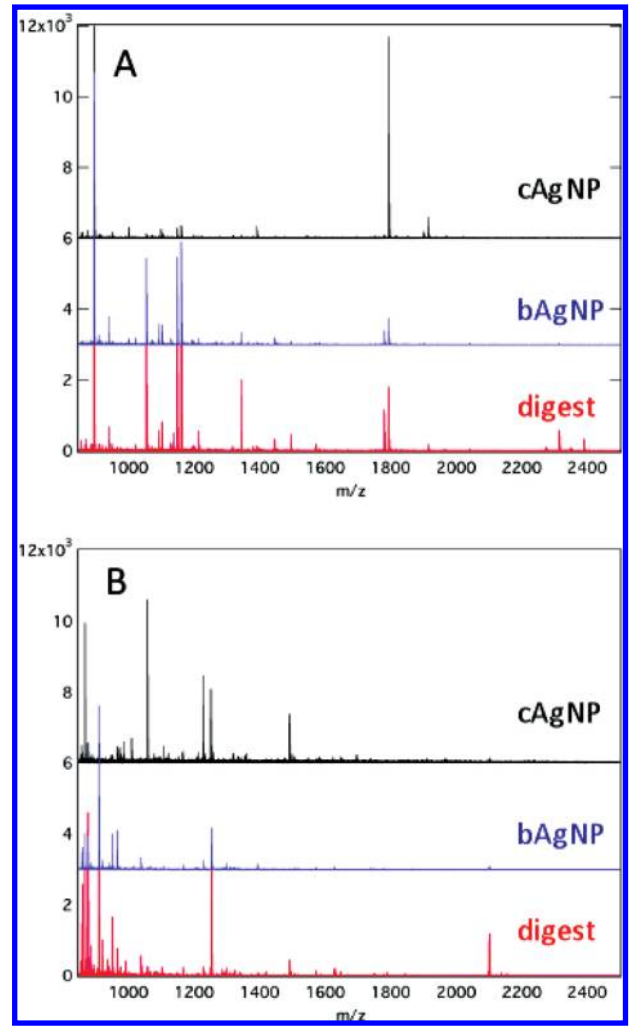

FIGURE 3. Normalized MALDI mass spectra from one experiment for (A) TNase trypsin-digested fragments bound to cAgNPs and bAgNPs with the spectrum of the unreacted tryptic digestion for reference and (B) TNase chymotrypsin-digested fragments bound to cAgNPs and bAgNPs with the spectrum of the unreacted chymotrypsin digestion for reference.

enzymatic digestions are retained in AgNP pellets after washing (data not shown). This enrichment of some protein fragments (and the absence of others) in a competitive environment suggests certain regions of TNase have enhanced affinity for AgNPs (Figure 3A and 3B). Reproducibly high-binding TNase fragments (defined as peaks at least 10% the height of the base peak) from at least two experiments are shown in Table 1. High-binding TNase fragments specific only to one type of AgNP reinforce the prior observation that

TABLE 1. Peptides Identified in MALDI Mass Spectra To Have High Affinity for Either cAgNPs or bAgNPs[a]

protein	sequence	pI	mass (Da)	hydrophobic ratio	binding/adduct formation	
					cAgNP	bAgNP
TNase	HLPEPFR	6.75	895	0.57	adduct	yes
	KHLPEPF	6.75	867	0.57	adduct	adduct
	TIEQITRETY	4.53	1253	0.20	adduct	yes
	NIFGYQYTIPTHQGR	8.60	1794	0.27	adduct	yes
	GNFDLEGLER	4.14	1149	0.40	yes	adduct
	DWTIEQITR	4.37	1161	0.33	no	yes
	AVEIGSFLLGR	6.05	1161	0.55	no	yes
	GLTFTYEPK	6.00	1055	0.33	yes	adduct
AdhP	HHHHGMASMTGGQQMGR	9.78	1853	0.23	adduct	no
	IRPGQWIAIYGLGGLGNLALQYAK	9.70	2572	0.46	adduct	no
	AAFNSAVDAVR	5.88	1120	0.64	no	yes
BSA	DTHKSEIAHR	6.92	1193	0.20	yes	no
	QQCPFDEHVKL	5.32	1340	0.27	no	adduct
	EIARRHPY	8.85	1041	0.38	adduct	adduct
	YEIAR	6.00	927	0.40	no	adduct
	RHPEYAVSVLLR	8.75	1439	0.50	yes	adduct
	SRRHPEY	8.49	944	0.14	adduct	adduct
Cytc	IFVQKCAQCHTVEK	8.06	1633	0.36	adduct	adduct
	VQKCAQCHTVEKGGKHKTGPNL	9.31	2363	0.21	adduct	adduct
	TGPNLHGLFGR	9.44	1168	0.36	yes	no

[a] Only peptides found to be high binding in duplicate MALDI mass spectra have been presented. AdhP: alcohol dehydrogenase, propanol preferring. Cytc: horseheart cytochrome *c*. BSA: bovine serum albumin. Binding of peptide to AgNPs is indicated by "yes", lack thereof by "no", and binding with adduct formation by "adduct".

surface modifications can influence reactivity. In particular, two fragments (DWTIEQITR and AVEIGSFLLGR) only bind to the bare AgNPs. Binding of bAgNPs to the second fragment may explain the higher inhibition of TNase activity with bAgNPs vs cAgNPs: this peptide is located in close proximity to the active site of the enzyme (Figure S6, Supporting Information); thus, binding of the protein to AgNPs may sterically block access to the active site.

In addition, one high-binding protein fragment (NIFGYQYTIPTHQGR) contains an amino acid (Arg103) that is part of the enzyme's active site (*29*). Although the amino acid Arg103 is buried within the molecule, the bulk of the peptide containing Arg103 and binding to AgNPs is located on the protein surface. Binding to this peptide could therefore shield the active site (Figure S6, Supporting Information) or distort it so it no longer retains full enzymatic activity and may be a mechanism by which AgNPs inhibit activity.

We also performed similar experiments with fragments of three other proteins that have various biological functions to compare with the TNase results. Binding of these three purified proteins to AgNPs is expected based on their structural properties (e.g., Cytc and AdhP have metal-binding sites) or previous adsorption experiments (BSA has been used to stabilize AgNPs in high salt content medium (*11*) and has previously been shown to bind to Fe_3O_4 nanoparticles (*30*)). Their high-binding protein fragments are also identified in Table 1. Like TNase, some differences in binding to AgNPs depending on surface modification were observed for all three proteins.

Taken together, all of the high-binding protein fragments in Table 1 demonstrate that binding can occur across a wide range of pI (4.53−9.78), which suggests adhesion is not simply a function of electrostatic affinity. Moreover, the hydrophobic ratios of the high-binding protein fragments also vary quite significantly, suggesting that hydrophobic interaction may not be the driving force for AgNP binding either. Perhaps not surprisingly due to their different surface properties, this is in contrast to previous studies on hydrophobic polystyrene nanoparticles (*22*).

Table 1 also highlights certain high-binding peptides that are present with a mass shift of 107 Da which correspond to Ag adducts. Ag adducts indicate one Ag atom is bound to the protein fragment when it is detected in the MS and have been previously observed with peptides bound to AgNPs or silver substrates (*31, 32*) and with silver ions binding organic molecules (*33–35*). The presence of Ag adducts indicates either that the laser induces ionization/desorption of AgNPs during analysis, creating Ag^+ ions that then associate with peptides as weak, nonspecific complexes (as is common for ions present in salts such as Na or K), or that the peptides are tightly bound to surface-associated Ag atoms before laser-induced desorption of the peptide−AgNP conjugate. Because the presence of these adducts may provide clues into the binding strength/mechanism of protein fragments to AgNPs (i.e., this could determine if the protein fragments associate with trace amounts of Ag^+ ions in solution or with the AgNP surface), we investigated adduct formation with free as well as complexed Ag^+.

MALDI mass spectra of the protein fragments after incubation with AgNPs, a Ag^+-complexing agent (cysteine) or $AgNO_3$, an aqueous form of Ag^+, indicate Ag^+ ions are not the cause of Ag adduct formation (Figure 4). Cysteine has been shown to sequester available Ag^+ from solution into an aqueous complex without causing AgNP aggregation (*12*), leaving only AgNPs available to react with the protein fragments. The AgNP suspensions used here were previously shown through a number of methods to contain ∼1 mol % free Ag^+ (*12*). Upon addition of an equimolar amount of cysteine to the initial incubations, the formation of Ag adducts was not abolished, suggesting that free Ag^+ is not important for their formation. Furthermore, MALDI mass spectra of the same protein fragments reacted with $AgNO_3$ (a source of free Ag^+) and then rinsed of excess Ag^+ did not show any evidence for the formation of Ag adducts. These two observations suggest that Ag adducts are not caused by the formation of weak complexes with free Ag^+ in solution nor are they simply byproducts of the laser-induced ionization process. Instead, Ag adducts may indicate a strong, specific association of protein fragments with the AgNP surface. The reason that only some of the high-binding protein fragments form Ag adducts is not known, but it is possible that it reflects relative binding strengths with the AgNPs.

Possible binding mechanisms are also apparent when considering the reactivity of individual amino acids or

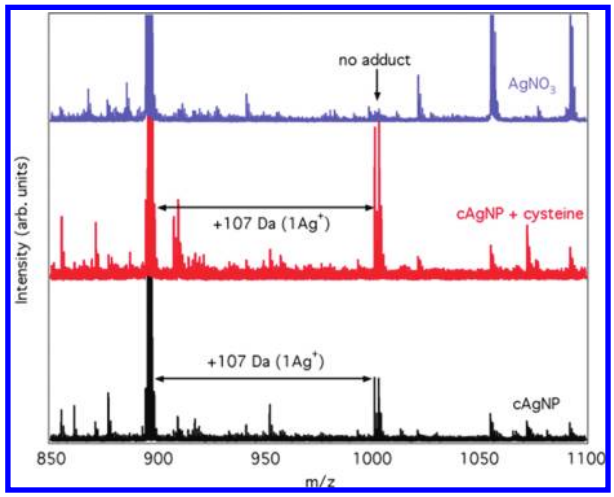

FIGURE 4. MALDI mass spectra of 895 Da TNase protein fragment and Ag adducts. The mass shift is evident when protein fragments are reacted with cAgNPs with and without the presence of cysteine. When these same protein fragments are reacted only with $AgNO_3$, no Ag adducts form.

functional groups. Several functional groups have previously been shown to be important for binding to Ag. For AgNPs, cysteine (*36*) and amine-bearing amino acids (*37*) contain the bond-forming residues. In addition, carboxyls (*36, 38, 39*) and aromatics (*36–38, 40*) were also found to be involved. One study alone identified sulfhydryl, carboxyl, aromatic, and imino groups across a wide range of proteins as responsible for binding to AgNPs (*41*), suggesting binding may be controlled not by a single amino acid but by specific sequences of amino acids.

Histidine is one residue known to be highly specific to metals in a wide range of metalloproteins; this specificity is exploited to purify recombinant proteins by attaching a histidine-rich tag onto a protein to bind to a Ni column. The AdhP construct that was used contained a His-tag sequence for purification, and indeed, a peptide containing this sequence (HHHHGMASMTGGQQMGR) was identified as a high-binding peptide to cAgNPs. In fact, many of the high-binding fragments contain one or more histidine residues despite its relatively low natural occurrence in proteins (~2.8% in all proteins). In Table 1, histidine comprises 5.6% of the total amino acids if the His-tag sequence in AdhP is ignored and 7.4% if it is included.

Some peptides in Table 1 share similarities to binding motifs previously identified in synthetic peptides by their strong binding to metal nanoparticles. For example, the TFTY motif in the 1055 Da fragment of TNase is similar to hydroxyl- and aromatic-rich sequences found in synthetic peptides with high affinity for AgNPs (*42*). Due to the small sample size of protein fragments listed in Table 1, we were unable to identify specific recurring binding motifs or sequence similarities across all peptides that have been observed for Ag or other metals using high-throughput screening methods (*42–44*).

Environmental Toxicity Implications. The strong binding of AgNPs to TNase resulted in the significant reduction of the enzymatic activity, suggesting that direct interaction of AgNPs with enzymes may lead to impaired metabolism. Although all forms of Ag are toxic at some level, previous observations that AgNPs are more toxic to bacteria than Ag^+ may be founded by the principle that protein binding to the nanoparticle surface changes the conformation or shields the active site of essential enzymes on the surface or within bacterial cells.

These observations have implications for understanding the ecotoxicity of AgNPs in aquatic systems, which is partially controlled by interactions with biomolecules. The high specificity of some protein fragments (evidenced by the presence of Ag adducts) may relate to previous studies showing that biomolecules induced AgNP dissolution and/or oxidation (*12*). The high affinity of some bacterial proteins also suggests that AgNP mobility in the environment may be limited once they come in contact with microorganisms. This fact could contribute to the lack of widespread toxicity of AgNPs to bacterial communities in natural sediments (*7*).

Acknowledgments

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Supporting Information Available

Additional methods details, characterization, SDS PAGE gels, TNase aggregation assay results, localization of peptides on the protein, and description of *E. coli* high-binding proteins identified from AgNP pellets. This material is available free of charge via the Internet at http://pubs.acs.org.

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