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Characterization of Nanomaterials in Metal Colloid-Containing Dietary Supplement Drinks and Assessment of Their Potential Interactions after Ingestion

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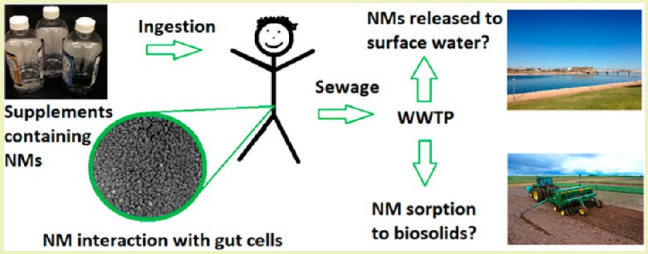
Supporting Information

ABSTRACT: Little information is available regarding the suitability of analytical methods to evaluate claims regarding the presence of engineered nanomaterials (NMs) in consumer products, their potential toxic effects to humans, or their life cycle after product use. This study was designed to assess the potential interactions across the life cycle of eight commercially available dietary supplement drinks from a single vendor, all purported to contain metal NMs. Analysis showed that all of the products contained metallic NMs with average diameters below 50 nm as determined by dynamic light scattering and transmission electron microscopy. The products' intended use is human ingestion; in order to examine potential human health effects after ingestion, we investigated the interaction of NMs in the drinks with an in vitro cell system that faithfully mimics human intestinal cells. After exposure to concentrations of NMs as low as 3.5 μg/mL, we found that the number of microvilli decreased relative to untreated controls for all drinks. From a life cycle perspective, consumption of drinks containing NMs will eventually result in sewer discharge of these NMs in feces. Screening tests for NM removal by biosolids in wastewater treatment plants (WWTPs) conducted using the NMs contained in supplement drinks showed variable removal of NMs, with the fractions removed ranging from (99 ± 27)% to (30 ± 0.05)%. The results showed that metal NM-based supplements may have an effect on the number of viable human intestinal microvilli and will likely enter the environment via either water or solids released from WWTPs.



KEYWORDS: *Dietary supplements, Electron microscopy, Nanoparticle, Silver, Silica, Toxicity, Colloidal, Food*

INTRODUCTION

With the recent increase in applications leveraging nanomaterials (NMs) for their novel properties, a wide range of consumer products has arisen to take advantage of the potential benefits surrounding "nano". One of the fastest growing sectors of nanoenabled products is in food and personal care products,[1] two product lines that involve direct human exposure to NMs as well as potential indirect release to the environment through waste disposal (e.g., sewage treatment, landfill). Despite these trends in the use of NMs, few studies avail that validate manufacturer claims regarding the NMs in the products or work with actual food products to assess toxicity or environmental fate.

The use of NMs in food can be classified based on the NM chemical composition or manufacturing process. Top-down processes (e.g., milling, grinding) are used to decrease the particle size and increase the specific surface area of organic constituents in food (e.g., wheat flour,[2] green tea[3]). Nanoscale liposomes are used to encapsulate hydrophobic nutrient supplements. Food packaging may also contain NMs for increased tensile strength, controlling gas permeability, controlled released of nutrients, and antimicrobial purposes.[4] The addition of NMs directly to food often has similar aims, such as oxidation protection, delivery of nutrients,[4] anticaking,[5] and antimicrobial[6] effects. With increased use in food and other consumer products, NMs will be released into the environment, with currently unknown environmental and human health effects.

The use of NMs in food and personal care products leads to human exposure through inhalation, ingestion, dermal contact, and as a combination of all pathways.[7] The daily use of these products also cause the release of NMs to sewer systems during their intended uses.[8−10] For instance, fabric, a plush toy, and cleaning products all claiming to contain silver NMs showed a

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potential dermal exposure of silver NMs to humans, and release of silver to synthetic urine.[10] The silver contained in urine would enter the sewer system and wastewater treatment plants (WWTPs).[11] In a life cycle assessment view on these products, there is growing concern regarding the potential negative effects of NMs and their removability in WWTPs.[12]

Nanotoxicology data exists on NMs in both in vivo and in vitro tests.[13−18] Toxicity studies focused on dermal and inhalational exposure of NMs to human cells have demonstrated their potential for causing cellular damage and reactive oxygen species production,[19−22] while studies focused on ingestion of NMs have primarily investigated their fate and transport in the gut.[23,24] Food grade silica NMs were found to arrest the Caco-2 cell cycle and inhibit cell growth during in vitro testing.[25] Silica is a commonly used NM in food.[5] Furthermore, the extent of TiO_2 NM incorporation into food has been tested,[1] and it is likely that these and other NMs will increase in use in the near future. As a potentially toxic metal to bacteria and aquatic organisms[26−28] used in popular nano-enabled products, nanosilver has been studied often, yet data on only a few commercial products exist.[11,29,30] Most information on the presence of metallic NMs in consumer products is on nanoscale TiO_2 or SiO_2 in foods.[1,5] Varied conclusions on the toxicity of nano TiO_2 and SiO_2 exist, depending on the cell lines or organisms investigated.[19,31−33]

The sustainable use of NMs involves balancing their benefits to society with their potential negative impacts on human and environmental health.[34] An understanding of NM life cycle after use and release into the environment is key to this assessment. In the case of NM-containing foods and drinks, a major portion of the life cycle will be their fate after excretion by consumers. Previous studies showed TiO_2 NM removal by a WWTP was in excess of 90%,[35] leaving 10% or less of the NMs in water to be discharged as effluent to riverine environments. A range of NMs varied in their extent of association and settling with biomass, from 88% for fullerenes to 13% for fullerols,[36] indicating the importance of transformations and surface properties for NM fate.

To fill the knowledge gaps identified above, we examined 8 commercially available dietary supplement drinks claiming to contain different metallic NMs for the purpose of improving human health and function for a variety of organs. Although NM-containing drinks may represent a niche product in the context of dietary supplements, we selected these products to represent foods and beverages that contain freely dispersed NMs. The goals of this study were (1) to demonstrate the ability of advanced analytical techniques to detect and characterize metallic engineered NMs in real commercial products, (2) to assess the potential effect of these nanoenabled products on a human intestinal cell type it may interact with as a result of intended use, Caco-2 cells, (3) assuming passage of excreted NMs to WWTPs, to determine the fractions of NMs in these products that may accumulate and settle with biomass or may be discharged to the environment in WWTP effluent, and (4) interpret findings from a life cycle perspective. Overall, this study adds to the limited available literature on NMs in food products and simultaneously assesses the potential toxicity and fate during selected time points in the life cycle of nanoenabled products.

EXPERIMENTAL SECTION

Eight representative drink products intended for human consumption and claiming to contain a range of metals (Ag, Au, Cu, Ir, Pd, Pt, Si, and Zn) in colloidal form were obtained from a single company, Purest Colloids, Inc. (Westampton, NJ).

All drinks were characterized in deionized water by dynamic light scattering using a Malvern ZetaSizerNano instrument (Worcestershire, UK). The zeta potential for each drink was measured without adjusting solution pH using a ZetaPALS zeta potential analyzer (Brookhaven, Holtsville, NY). A Philips CM 200-FEG transmission electron microscope (TEM) with energy dispersive X-ray spectroscopy (EDX) was used for imaging of particles and analysis of size and elemental composition. Samples were prepared for TEM analysis by drying a single drop of undiluted solution on a lacy carbon mesh on top of a copper grid. ImageJ was used to size NMs in TEM images, with at least 100 particles sized for each sample.

Sizing of NMs by single particle inductively coupled plasma mass spectrometry (spICPMS) was performed on a PerkinElmer NexION 300q ICPMS, following analytical and data processing methods described elsewhere.[37] This technique has been used previously to successfully size Au and Ag NMs.[38,39] Further information is available in the Supporting Information (SI).

The total metal concentration in each supplement was determined by digesting 5 mL of sample using 5 mL HNO_3 (Ultrex II, J.T. Baker) and 10 mL nanopure water for drinks containing Ag, Cu, and Zn. Aqua regia (3:1 HCl:HNO_3) was used in the place of HNO_3 for digestion of drinks containing Au and the platinum group elements Ir, Pd, and Pt. For the Si drink, tetramethylammonium hydroxide was used in place of the concentrated acids. The digestions were performed in glass beakers, with the exception of the Si digestion which used a Teflon beaker, on a hot plate at 200 °C until reduction of the volume to ∼5 mL. This solution was then diluted in a volumetric flask with 2% HNO_3 for analysis by ICPMS (Q Series iCAP, Thermo, Waltham, MA).

Total organic carbon (TOC) analysis was done using a total organic carbon (TOC) analyzer (Sunset Laboratories, Oregon, USA). Liquid samples (40 μL) were loaded on a quartz fiber filter (QFF), dried, and then analyzed for TOC.

The presence of crystalline particles was detected using X-ray diffraction (XRD) (X'Pert Pro Materials Research X-ray Diffractometer, PANalytical, Netherlands) with a Cu Kα source and quartz holder. Each sample was scanned from $2\theta = 35°$ to 50° or $2\theta = 22°$ to 40° (for silicon) to detect the characteristic peaks. Samples were prepared by filtering approximately 100 mL of solution through a 0.2 μm nylon filter. Particle retention was observed immediately by both color change of the filter (and filtrate) and by the decrease in flux.

To test for any potential impact of the supplement drinks NMs on human intestinal cells, exposure experiments with representative cells were performed. The human, brush border-expressing Caco-2 cell line was purchased from American Type Culture Collection (CRL-2102) at passage number 47 and maintained as described elsewhere.[40,41] Methodology for analysis of cells by scanning electron microscopy (SEM) has been described in detail elsewhere.[42] Microvilli on the cell surface were individually counted as described elsewhere.[42] Briefly, three random 1 μm^2 windows were generated per SEM micrograph, the numbers of microvilli were counted in each window, and the three regions were averaged. No fewer than three micrographs were taken per epithelium, and each experiment was conducted three independent times.

Further details regarding this biological assay can be found in the SI. A two-tailed Student's *t*-test was used to assess statistical significance in the difference of means for data sets.

To estimate the potential removal of NMs contained in the supplement drinks by wastewater treatment plants (WWTPs), a series of biosorption batch tests were conducted with the drinks on activated sludge as described previously.[43] The total suspended solid (TSS) concentration after washing was determined according to standard methods.[44] Further detail on biosorption tests can be found in the SI.

An aliquot of 5.9 mL clean biomass was spiked into a series of amber glass vials containing the supplement drinks and 1 mM of $NaHCO_3$ buffer solution. For the silica NM-containing drink, plastic vials were used to avoid potential Si interference from glass during subsequent ICPMS analysis. The final biomass concentration was 1000

mg TSS/L, which is in the lower range of activated sludge concentration in aeration tanks.[45] The dosing concentration of NMs in each vial was 1 mg/L (1000 mg/kg biomass), which represents the high concentration scenario of NMs in sludge treatment plants based on predicted values of 1.53−137 mg NMs/kg biomass.[46] Additionally, this concentration of NMs was used to be comparable with other biomass sorption studies on engineered NMs.[36,47] After mixing on a shaker table for 3 h, the biomass was gravitationally settled for 30 min. The digestion of supernatant was performed as listed above for the drinks' metal concentrations, with the addition of 3 mL H_2O_2 (Ultrex II, J.T. Baker) to oxidize any residual biomass before introduction to the ICPMS.

RESULTS AND DISCUSSION

Metal and Carbon Content Determination. Analysis of digested supplement drinks by ICPMS determined primary metal concentrations to be in the milligram per liter range, from 4.39 mg/L Pd to 102 mg/L Si (Table 1). For all but three drinks, the only metal above the minimum detection limit was the primary metal claimed by the manufacturer to be present as the active NM. These three drinks all contained Ag as a secondary metal: Ir drink (0.63 mg Ag/L), Pd drink (1.11 mg Ag/L), and Pt drink (1.30 mg Ag/L).

TOC analysis revealed that three drinks contained carbon: the Au drink was found to contain 55.6 mg C/L, Ag drink 3.1 mg C/L, and Si drink 4.5 mg C/L; all others had no apparent carbon content (no detect above background carbon). This carbon content may indicate the presence of either carbonaceous artifacts remaining from synthesis or an added organic compound (e.g., tannic acid or citric acid) used to stabilize the particles.

NM Sizing. Three analytical techniques were used to size NMs in the drink matrix, stated on the label to be deionized water. Representative TEM images for all materials can be seen in Figure 1. The NMs are all roughly spherical in shape. The NM size distributions from TEM analysis are shown in Figure 2, with averages summarized in Table 1. Size distributions generated by DLS are presented in Figure 2, with averages summarized in Table 1. The DLS size distributions all show a large second peak in addition to a peak near the number-based average diameter. Light scattering intensity increases as a sixth power of the particle size,[48] so the presence of much larger particles in solution will create an intense second peak. The number of particles in these larger peaks was less than 5% of total particles in all cases, so the number-based average is representative of the majority of particles in the samples. The presence of some very large (diameters of micrometers) particles found during TEM analysis may explain the second peak observed by DLS. The majority of average particle sizes analyzed by DLS and TEM from each drink confirmed colloid sizes reported by the manufacturer, although some very large (on the order of a couple micrometers) particles were found in Si samples.

Analysis of the drinks by spICPMS allowed particle detection for the primary metal NMs in each drink (Figure S1 shows this for the Au and Pd examples). In the case of analyzing the supplement drink NMs, the average size of the particles was too small to be accurately sized by spICPMS. The observed particle detection events are likely attributable to the coincidence effect, where multiple particles are detected in a single dwell time.

EDX and XRD Characterization. EDX results agreed with elemental analysis by ICPMS, confirming the major metal present in each drink. Representative EDX spectra are shown for two beverages (Ir and Pd NM drinks) in Figure 3, with the

Table 1. Summary of Supplement Drink NM Characterization by ICPMS, DLS, and TEM[a]

stated element in drink	Ag	Au	Cu	Ir	Pd	Pt	Si	Zn
primary (secondary) element present by ICPMS	Ag	Au	Cu	Ir (Ag)	Pd (Ag)	Pt (Ag)	Si	Zn
concentration of major element (mg/L)	21.3 ± 1.39	6.11 ± 1.18	14.4 ± 0.67	20.0 ± 2.37	4.39 ± 0.50	17.2 ± 1.83	102 ± 11.1	5.18 ± 0.42
NM diameter reported by manufacturer (nm)	<0.65	~3.2	NR	20	NR	15−20	~6	20
hydrodynamic diameter by DLS (nm)	1.46 ± 0.04	1.17 ± 0.50	ND	17.3 ± 2.79	22.4 ± 4.15	44.0 ± 6.89	9.66 ± 0.19	ND
polydispersity index by DLS	0.85 ± 0.10	0.25 ± 0.04	1.0 ± 0.0	0.37 ± 0.05	0.38 ± 0.02	0.41 ± 0.05	0.19 ± 0.02	1.0 ± 0.0
diameter by TEM (nm)	10.9 ± 4.51	5.35 ± 2.40	10.2 ± 2.52	11.6 ± 3.41	4.18 ± 1.20	4.55 ± 1.37	8.73 ± 1.57	17.1 ± 4.32
particle morphology by TEM	spherical	spherical	fused spheroids	largely amorphous	largely amorphous	irregular	spherical	irregular
zeta potential (pH)[b]	−7.93 ± 8.88 (8.9)	−1.09 ± 4.13 (6.2)	11.38 ± 3.37 (7.8)	−11.86 ± 4.72 (7.6)	−19.71 ± 4.78 (4.9)	−34.38 ± 4.99 (4.7)	−2.87 ± 7.65 (9.1)	2.33 ± 9.72 (5.5)

[a]NR = not reported by manufacturer. ND = not detected by instrument. Error measurements are standard deviations of replicate samples: 3 replicates for DLS measurements, 8 replicates for zeta potential, and at least 100 particles measured for TEM average size. [b]The pH of the supplement drink at which zeta potential was measured is indicated in parentheses.

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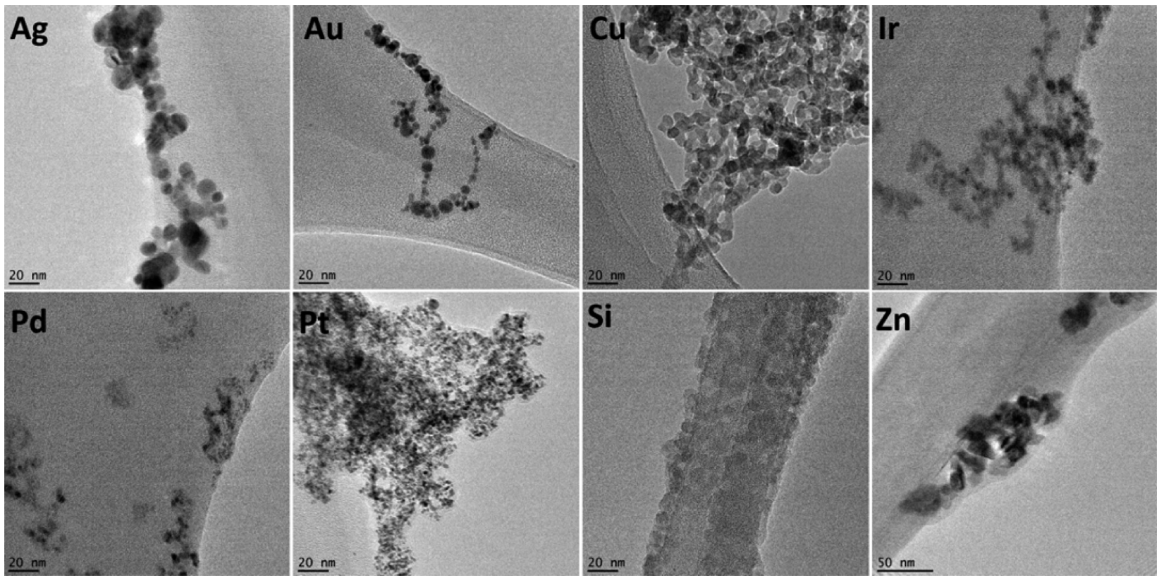

Figure 1. Representative TEM images of NMs found in supplement drinks.

spectra for the remaining six NMs shown in Figure S2. Several NMs (Cu, Si, and Zn) had a large EDX peak for oxygen and are likely to be present in an oxide form. This oxygen could also be associated with carbonaceous molecules adsorbed to the surface, as revealed by TOC analysis.

SI Figure S3 shows the XRD spectra for the supplement drink NMs. XRD analysis revealed the presence of a crystalline structure for silver, iridium, palladium, platinum, gold, and zinc. No obvious crystalline structure was observed for silicon and copper. This suggests that the silicon sample is amorphous, possibly SiO_2, and the copper sample is presumably in the form of an amorphous copper oxide, which is supported by the green tint of the solution.

Impact of Drinks on Intestinal Microvilli. The metal NM-containing supplement drinks were used for intestinal cell exposure tests, to assess potential effects of these materials on the human gut. In human intestinal cells, the brush border, composed of many individual microvilli, exists to provide additional cell surface area. The unique array of bundled actin filaments that form individual microvilli of the brush border provides for an archetypical structure that is evolutionarily conserved from invertebrates such as worms, to vertebrates including humans. When viewed by SEM, the brush border, composed of many individual microvilli, appear as finger-like projections emanating from the cell surface. Each microvillus has an axial diameter of ∼100 nm. As shown in SI Figure S4, untreated control brush borders have 40 ± 6 microvilli/μm^2, which is consistent with other reports,[41,49,50] and appeared to stand straight off the cell surface in an orientation referred to as erect.[51−53] However, after exposure to 350 ng/mL of the dietary supplement drinks used in this study both the organization of the brush border and the number of microvilli per unit area decreased for many, but not all, of the brush borders exposed to the NM-containing supplements (SI Figure S5).

Exposing specimens to low (350 ng/mL) concentrations of NMs as shown in SI Figure S4 makes it difficult to visualize which regions are in contact with the material as NMs. In order to more clearly delineate the extent of disruption and determine which material has marked effects on the brush border, epithelia were exposed to drink supplement concentrations of 3.5 μg/mL. In this series of experiments untreated control brush borders contained 50 ± 2 microvilli/μm^2 (Figure 4A) and maintained the erect, archetypical organization as was shown in numerous reports.[51−53]

After exposure to supplement drinks at the higher drink supplement concentration of 3.5 μg/mL, all of the test materials elicited a significant ($p < 0.05$, $n = 6$) decrease in the number of brush border microvilli compared to controls (Figure 5). The lower dose of 350 ng/mL, by comparison, resulted in significant decreases in microvilli/μm^2 for Au, Ir, Pt, Si, and Zn, as shown in SI Figure S4. Additionally, many microvilli appear to be going limp or clumped, indicating an effect due to the supplement drinks.

To date, within the experimental paradigm of brush border disruption, no published study has employed NM concentrations as low as 350 ng/mL. The concentration of 350 ng/mL is equal to 100 ng/cm^2 (based on the method of NM application in this study) that the cells could be exposed to if sedimentation predominated.[54,55] However, since most of the NMs employed in the present study had small primary particle diameters, it is unlikely that sedimentation of the material was a major causal agent for brush border disruption in this system. In the present investigation, some, but not all, of the NMs resulted in effacement of the brush border. Whether or not brush border disruption occurs in vivo remains to be elucidated, since the in vivo mucosal epithelium is turned over within a week.[56] Directions on the bottles of the eight supplements recommended that the drinks should be taken daily. It is difficult to extrapolate the effects observed after a single "acute" in vitro exposure compared to a "chronic," albeit potentially less concentrated dose in vivo recommended by the directions. Together, these data indicate the need to investigate further in vivo effects in models that accurately emulate the human gut, since a loss in the number of microvilli could result in pathological states such as malabsorption or diarrhea.

Biomass Sorption of Supplement NMs. The metal NM-containing supplement drinks were used for biomass sorption tests to assess the potential extent of their removal in biosolids at WWTPs. Figure 6 shows the percent removal of Ag, Au, Cu, Ir, Pd, Pt, Si, and Zn metal from the liquid phase after exposure of supplement drinks to one biomass dose for 3 h of agitation.

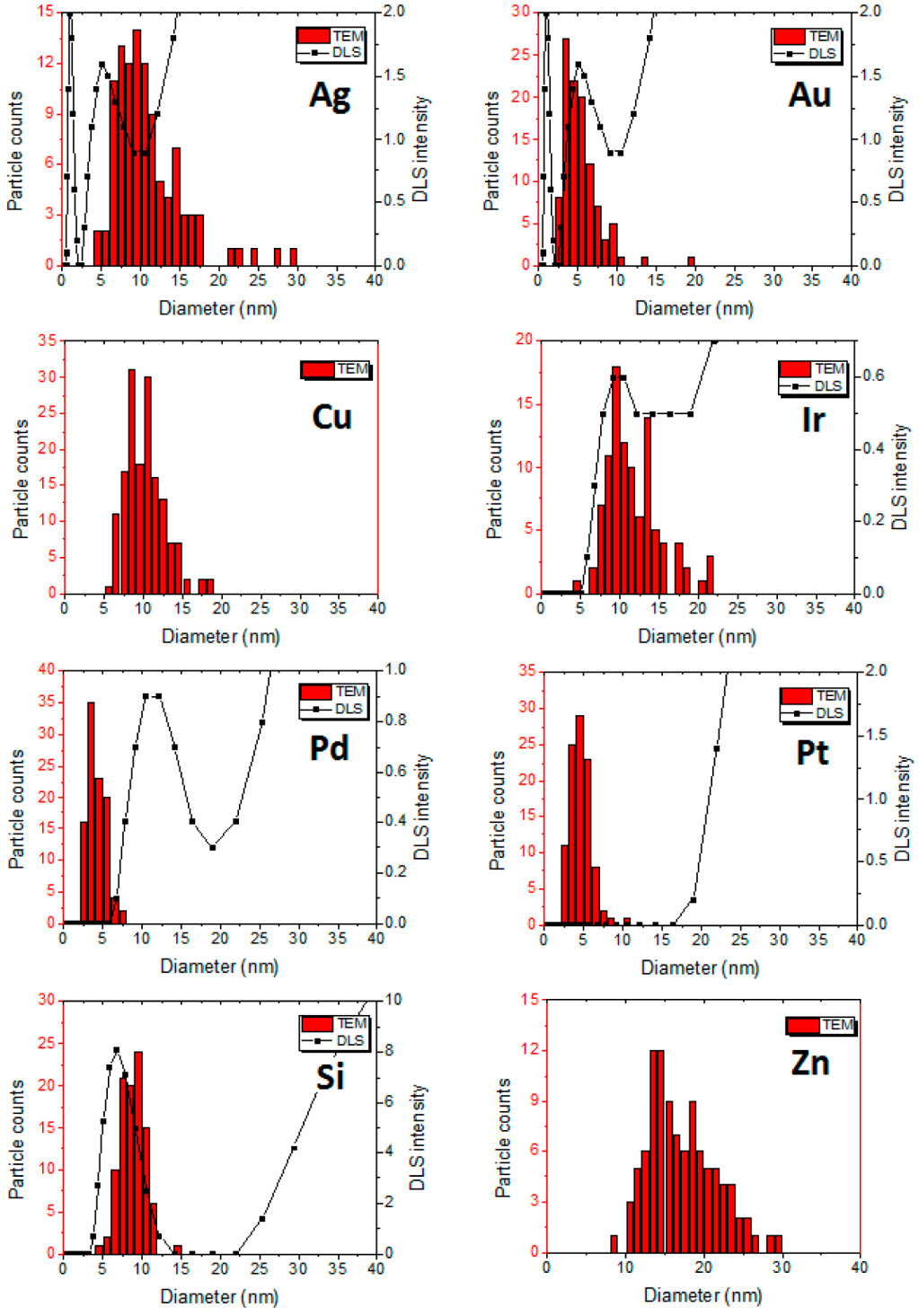

Figure 2. Size distributions of NMs in supplement drinks measured by DLS and TEM. Particles were not detected by DLS for Cu- and Zn-containing drinks.

The percent removals were in the range of 30 ± 5% to 99 ± 27% for the eight types of NMs. A 50 nm citrate-coated Au NM was included in the biomass sorption experiments as a positive control and was removed from solution below detectable limits. A typical WWTP operates with 1000−5000 mg/L total suspended biomass per liter. For the NM-containing beverages, in the presence of the 1000 mg/L biomass dose, 98 ± 27% of Au NMs were removed, while the removal percentages for Ag, Cu, and Zn NMs ranged from 82 ± 5% to 90 ± 10%. The removal percentage of Pt was 56 ± 9%, and the lowest removal percentages were observed for Ir, Pd, and Si NMs with an average removal rate of 35%. Our results showed that platinum group element−containing NMs such as Ir, Pd, and Pt may only be partially removed by activated sludge WWTPs. Differences in NM coating or functionalization have been shown to affect removal by biomass,[43] and NM dissolution may play an important role as well. Biomass zeta potential is negative near the neutral pH used in this sorption experiment, which may explain the differences in removal. To explore the potential relationships between NM surface charge and

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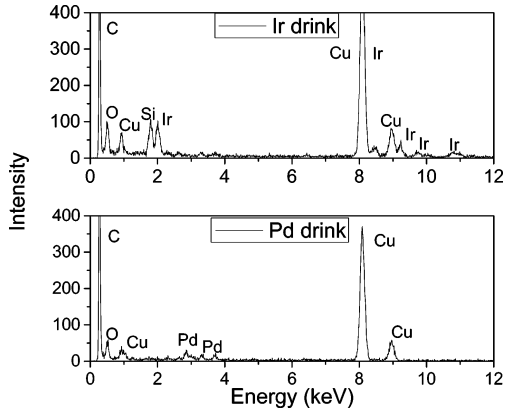

Figure 3. Representative EDX spectra of NMs from drinks claiming to contain Ir and Pd colloids. The remaining EDX spectra can be found in the SI (Figure S2). The Cu and C peaks are due to the TEM grid material.

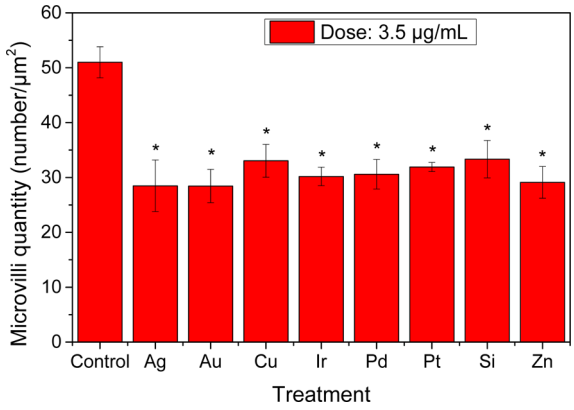

Figure 5. Quantification of Caco-2 cell microvilli after exposure to supplement drinks at a dose of 3.5 μg/mL. All drinks had a significant effect on the number of microvilli present compared to the untreated control. The asterisks (*) indicate statistically significant difference from control ($p < 0.05$).

tendency to distribute onto the biomass surface, zeta potential of the NMs in the drinks were measured (Table 1). Zeta potential ranged from +11 to −34 at the pH levels in the drinks (4.66 to 9.06). The particles which had more negative zeta potentials (Ir, Pd, Pt) were not removed effectively (Figure 6), while those with zeta potentials close to or above zero were removed almost completely. The exception to this is the Si NM, which has been previously shown to be stable when other NMs are not.[57]

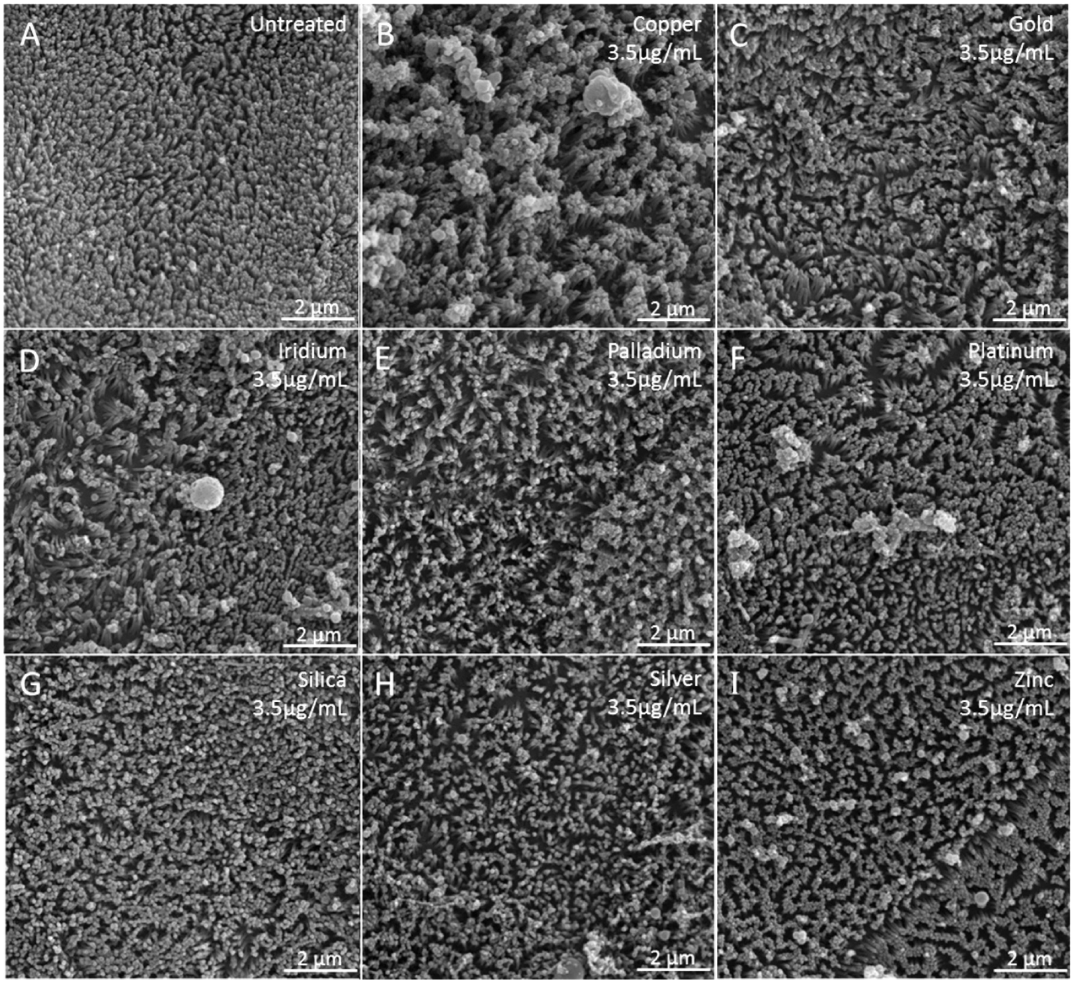

Figure 4. SEM images of microvilli exposed to 1 μg/cm^2 NMs (3.5 μg/mL), except "A", which is an untreated control. After exposure to NMs from supplement drinks, both the normal organization and the number of microvilli changed compared to untreated controls. Large spherical particles (>250 nm) are membrane blebs.

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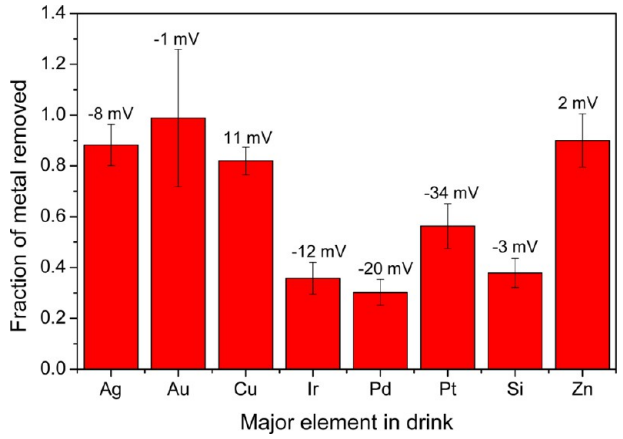

Figure 6. Fraction of each metal removed by settling with biomass in DI water and 1 mM $NaHCO_3$ buffer. Zeta potentials measured in the drinks are given for each NM (Table 1), rounded to the nearest millivolt.

In other work, it has been shown about 80−90% of functionalized silver NMs were removed from the liquid phase in the presence of 50−400 mg/L biomass,[36] which matches well with our present results. The Au, Cu, and Zn NMs showed a similar removal trend likely due to the same removal mechanism as Ag. However, the Ir, Pd, Pt, and Si NMs showed lower removal percentage than other four NM suspensions, which could be due to a different stabilizer or capping agent on those particles. However, an uncoated SiO_2 NM did not flocculate in wastewater for primary treatment while coated SiO_2 nanoparticles were removed.[58] Possibly the Si supplement drink contained a similarly uncoated SiO_2 particle and thus only 30% of them were removed by sorption to biomass.

CONCLUSIONS

Few studies have reported data on detection, characterization, or quantification of NMs in food and personal care products. To the best of our knowledge, the present study is the first to report characterization of metal NM-enabled dietary supplement drinks meant to be directly ingested. These drinks were characterized by a variety of techniques and were found to contain metal NMs in the 1−100 mg/L concentration range. The potential effects of these supplement drinks after exposure to an in vitro model of human intestinal cells was analyzed, and the results suggested a significant reduction in quantity of microvilli and loss of the normal erect morphology. In this study, the "subtle" (i.e., nonlethal) effect of brush border disruption was observed after exposure to the supplement drinks. Sorption tests using metal NMs from supplement drinks showed a variable extent of NM association with biomass, suggesting there may be a difference in NM removal in WWTPs. Overall, this work contributes to the literature on metal NMs in food products and examines the potential interactions at key points in the life cycle of these NMs.

ASSOCIATED CONTENT

Supporting Information

Additional experimental descriptions and graphs. 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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